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Estudo da contribuição molecular e celular do perióstio na craniossinostose da síndrome de Apert

Study of the molecular and cellular contribution of the periosteum to the craniosynostosis in Apert syndrome

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*A todos que não só não se intimidam pelos
desafios, como acreditam que esta é a parte
mais divertida e estimulante da Ciência.*

*“Faço histórias para crescer, não para
acrescentar.” Laerte*

“The main thing that I learned about conspiracy theory is that conspiracy theorists actually believe in a conspiracy because that is more comforting. The truth of the world is that it is chaotic. The truth is, that it is not the Jewish banking conspiracy or the grey aliens or the 12 foot reptiloids from another dimension that are in control. The truth is more frightening, nobody is in control. The world is rudderless.”

Alan Moore, in: “The Mindscape of Alan Moore” (2003)

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I. Introdução geral

O crânio

O crânio é formado por estruturas anatômicas arranjadas em uma rede física na qual o resultado final da morfogênese – baseada em alterações no tamanho e no formato das estruturas anatômicas (Bruner, 2007) – é o fruto das pressões e tensões associadas a órgãos em expansão (como o encéfalo), ligações conectivas (como a dura-máter e o perióstio), suturas contactantes, deslocamentos ósseos e influência muscular. Cada componente do crânio interage diretamente com as estruturas vizinhas gerando um sistema complexo em que toda a organização não é meramente uma soma de cada processo único (Bruner, 2007). Tais interações ocorrem em diferentes níveis, seja molecular, celular, tecidual, ósseo ou de órgãos. Nos tetrápodos, o crânio é formado de dois componentes principais: o viscerocrânio e o neurocrânio (Morriss-Kay, 2001; Morriss-Kay & Wilkie, 2005) (Figura I.1).

O viscerocrânio (correspondente à face) é onde se localizam a mandíbula, as estruturas de suporte a ela e outros elementos derivados do arco branquial e é derivado principalmente da crista neural (Kuratani et al., 1997). O neurocrânio inclui a caixa craniana e cápsulas sensoriais associadas (nasal, óptico eótico) e é principalmente de origem mesodérmica (Kuratani et al., 1997). Os ossos que fazem parte tanto do viscerocrânio quanto do neurocrânio, excluindo a caixa craniana, são originados de ossificação endocondral (Morriss-Kay & Wilkie, 2005; Colnot, 2009). A caixa craniana difere das outras estruturas do crânio por seus ossos serem originados de ossificação intramembranosa, ou seja, sem precursor cartilaginoso (Franz-Odenaal et al., 2006). As estruturas que a formam tem origem mesodérmica e de crista neural (Jiang et al., 2002). A região occipital é o componente mais caudal do crânio, formado por ossos endocondrais derivados de tecidos somáticos mesodérmicos (Morriss-Kay, 2001). No presente trabalho, o enfoque será dado ao conhecimento atual de crânio de mamíferos e, em específico, à caixa craniana, parte do neurocrânio. A estrutura óssea da caixa craniana é formada por oito ossos planos e irregulares – incluindo um frontal, dois parietais, um osso occipital, um esfenóide, dois temporais e um etmóide – rigidamente unidos pelas suturas cranianas, delineando a cavidade do crânio onde se aloja o encéfalo (Figura I.1). Nos mamíferos, diferente da maioria dos peixes, anfíbios e répteis, o crescimento do neurocrânio não é contínuo, terminando num período equivalente ao de maturação sexual (Morriss-Kay & Wilkie, 2005).

Figura I.1. Anatomia do crânio humano. Crânio humano adulto em diferentes orientações no qual o viscerocrânio está evidenciado em azul e o neurocrânio em verde. Os principais ossos estão indicados. (Modificado de Putz & Pabst, 2001).

Origem embrionária do crânio

A crista neural e a mesoderme são os tecidos embrionários que darão origem a todas as estruturas ósseas do crânio.

As células da crista neural se originam da neuroectoderme, localizada entre o tubo neural e as margens livres da dobra neural, em torno do 20º dia de gestação em humanos. Elas sofrem transição epitélio-mesenquimal, na qual perdem a adesão célula-célula e sofrem rearranjos no citoesqueleto, que levam a mudanças morfológicas, possibilitando a delaminação e emigração do neuroepitélio em direção a diferentes estruturas no embrião (Trainor & Nieto, 2003; Sauka-Spengler & Bronner-Fraser, 2008). Há subpopulações distintas de células da crista neural: as células da crista neural do tronco originam melanócitos e gânglios; as células da crista neural vagal e sacral formam gânglios entéricos; células da crista neural cardíaca originam o tecido conjuntivo dos grandes vasos; e, por fim, há as células da crista neural cefálica, que apresentam um repertório de diferenciação mais vasto que os demais grupos, e são capazes de originar cartilagem, osso, melanócitos, neurônios craniais, células da glia e tecido conjuntivo da face, além de constituírem os arcos branquiais e formarem as células do timo, os odontoblastos e primórdios dentais (Gilbert, 2006).

As células da crista neural cefálica migram da porção anterior, média e posterior do encéfalo em correntes migratórias distintas. As células que migram para as regiões frontonasal e primeiro arco brânquia contribuem para a formação do crânio (Morriss-Kay & Wilkie, 2005). Analogamente, as células mesenquimais mesodérmicas que darão origem a estruturas cranianas migram da linha primitiva para a região craniana do embrião (Lawson & Pedersen, 1992). Entretanto, as células da crista se mantêm separadas das células mesodérmicas.

As células da crista neural migram sob a superfície ectodérmica, formando uma passagem entre este tecido e o mesênquima mesodérmico (Chan & Tam, 1988) em direção ao primeiro arco branquial, que, conseqüentemente, sofre uma grande expansão (Morriss-Kay & Wilkie, 2005). Elas também migram entre a superfície ectodérmica e a parte mais rostral do prosencéfalo, o qual se expande rostralmente para formar a região do telencéfalo e não é associado ao mesênquima mesodérmico. No estágio de 23 somitos, que em humanos é em torno do 28º dia de gestação, a migração é completada e um limite claro entre tecidos derivados de crista neural e derivados da mesoderme é formado, o que sugere uma interação de repulsão agindo para evitar a mistura das duas populações celulares (Cohen, 2000, Morriss-Kay & Wilkie, 2005)

Figura 1.2. Origem embrionária das estruturas ósseas do crânio. Crânio humano de recém-nascido em diferentes orientações no qual os ossos de origem mesodérmica estão evidenciados em azul e os de origem da crista neural em rosa. As principais suturas cranianas estão indicadas. (Modificado de Putz & Pabst, 2001).

O osso frontal, osso esfenóide, parte do osso interparietal, parte do osso temporal, o mesênquima das suturas interfrontal e coronal e parte da sutura sagital são de origem da crista neural (Jiang et al., 2002). A dura-máter que recobre o prosencéfalo e sob os ossos frontal e parietal também é de origem de crista neural. Já os ossos parietais propriamente ditos e as meninges que recobrem o mesencéfalo e o rombencéfalo são de origem mesodérmica (Rice, 2008) (Figura I.2).

Formação e fusão das suturas cranianas

A principal característica do crânio humano é o formato resultante do lento e neotênico desenvolvimento craniano combinado a um crescimento rápido e hipermórfico do encéfalo (Zollikofer & Ponce de Leon, 2010), de forma que o crescimento do neurocrânio acompanha o do encéfalo. Como supracitado, os ossos da caixa craniana são unidos por tecido fibroso denominado de suturas e até que se atinja a maturação sexual, as suturas desempenham um papel importante no indivíduo por (1) permitirem a deformação do crânio ao nascimento, (2) absorver impactos mecânicos, protegendo o tecido osteogênico da sutura (Jaslow, 1990; Persson, 1995) e (3) ser o principal centro de crescimento do crânio, durante o aumento do encéfalo, regulando o balanço entre proliferação e a diferenciação das células osteogênicas precursoras (Wilkie, 1997; Slater et al., 2008). Durante o desenvolvimento, as suturas passam de limites lineares entre os ossos para complexas estruturas interdigitadas de dimensões fractais não íntegras (Miura et al., 2009). As suturas eventualmente se fundem e a idade de fechamento de cada sutura está detalhada na Tabela I. 1. Vale ressaltar que a sutura metópica normalmente é obliterada já por volta do terceiro ano de vida, e persiste em 10% ao longo da vida (Cohen, 2000), ao contrário das outras suturas que só iniciam a fusão na vida adulta, o que pode ser explicado pelo fato de ser a única de origem exclusiva da crista neural (Morris-Kay & Wilkie, 2005).

Tabela I. 1. Idade de fechamento de suturas cranianas em humanos (Retirado de (Cohen, 2000)).

Sutura craniana	Idade de início de fechamento (anos)
Metópica	2
Sagital	22
Coronal	24
Lambdóide	26
Esquamosal	35-39
Esfenofrontal	22
Esfenoparietal	29
Esfenotemporal	28-32
Masto-occipital	26-30

A formação intramembranosa dos ossos do neurocrânio se inicia do desenvolvimento de blastemas mesenquimais no segundo mês de gestação humana (Langille, 1994; Aubin et al., 1996; Cohen, 2000; Opperman, 2000), cujas células começam a diferenciar e depositar matriz extracelular composta principalmente de colágenos e outras proteínas e proteoglicanas relacionadas a osso (Ninomiya et al., 1990) que mineralizam. A ossificação intramembranosa decorre radialmente a partir destes focos mesenquimais (Alberius et al., 1992). As bordas de cada osso estão largamente separadas e vão se aproximando, até que se confinam ou sobrepõe, formando as suturas (Opperman, 2000). Durante o desenvolvimento das suturas, as frentes ósseas crescem e se expandem, invadindo e recrutando o tecido mesenquimal interveniente. Por volta do 50º dia de gestação em humanos, começa a separação do mesênquima em duas camadas pelos ossos em expansão: o perióstio na parte externa e dura-máter na porção interna (Artun et al., 1986).

Assim a sutura craniana é um complexo formado pelo perióstio sobrejacente, as frentes osteogênicas das placas ósseas, o mesênquima interveniente e a dura-máter subjacente (Figura I.3). Muitos experimentos têm fornecido evidências para a potencial influência da dura-máter e do perióstio sobre o fechamento das suturas cranianas. Foi observado em ratos e coelhos que a remoção da dura-máter leva a aceleração ou atraso no fechamento da sutura, dependendo de qual sutura a dura-máter é retirada (Opperman et al., 1993; Roth et al., 1996; Levine et al., 1998). Da mesma forma, a excisão do perióstio diminui a calcificação de defeitos cranianos em modelos animais (Hopper et al., 2001; Ozerdem et al., 2003).

Craniossinostoses

O termo “craniossinostose” se refere ao processo de fusão prematura das suturas do neurocrânio. Ela principia em um ponto e se espalha ao longo da sutura, podendo acometer uma, muitas ou todas as suturas e pode se estabelecer tanto no período pré- quanto pós-natal (Cohen, 2000). Ela é uma anormalidade craniofacial congênita comum e sua prevalência é estimada em 1 a cada 2.500 nascidos vivos (Cohen, 2000; Passos-Bueno et al., 2008). Como o crânio não pode expandir perpendicular à sutura fusionada, ele compensa crescendo na direção perpendicular à sutura aberta, fornecendo o espaço necessário para o cérebro em crescimento, mas resulta em uma forma anormal da cabeça e, às vezes, características faciais anormais (Cohen, 2000). As craniossinostoses são etiologicamente e patologicamente heterogêneas, mas, de acordo com estudos genéticos, elas podem ser classificadas como não sindrômica (ou isolada) e sindrômica (Cohen, 2000; Passos-Bueno et al., 2008).

São denominadas craniossinostoses não sindrômicas aquelas em que a fusão da sutura é o defeito primário no indivíduo, embora sintomas secundários (por exemplo, manifestações neurológicas e oftalmológicas) ocorram como consequência da sinostose da sutura (Passos-Bueno et al., 2008). Elas correspondem a 70% dos casos (Lajeunie et al., 1995; Passos-Bueno et al., 2008). Quando a fusão prematura das suturas ocorre concomitantemente a outros defeitos morfogênicos primários, a craniossinostose é classificada como sindrômica (Passos-Bueno et al., 2008).

Em estudo que acompanhou o atendimento genético a craniossinostoses por 10 anos, Wilkie e colaboradores reportam que o diagnóstico genético foi obtido em 21% dos casos, dos quais 86% eram causados por mutações em um único gene e 15% por anomalias cromossômicas (Wilkie et

Figura 1.4. Anatomia e morfogênese das suturas. (A) Composição anatômica por diversos tecidos da porção superior do crânio, do escalpo à pia-máter. (B) Representação diagramática dos estágios da morfogênese da sutura: Os sinais indutivos decorrentes das frentes ósseas (amarelo/laranja) se aproximando permitem que elas desviem uma da outra, sem obliteração da sutura (verde). Estes sinais são independentes de sinais de dura-máter ou osso. Quando as frentes ósseas sobrepõem uma a outra, sinalização proveniente do periósteo (rosa) e da dura-máter (vermelho) mantém a presença da sutura recém-formada (verde). Os ossos tornam-se espessados por depósito de osteóide e mineralização de novo na superfície periosteal, até que a sutura é fusionada.

al., 2010, Johnson & Wilkie, 2011). Os genes mais frequentemente mutados foram: *FGFR2* (32% de todos os casos com diagnóstico genético), *FGFR3* (25%), *TWIST1* (19%) e *EFNB1* (7%) (Johnson & Wilkie, 2011). Outros genes com mutações causativas bem estabelecidas para craniossinostoses, são *FGFR1* (síndrome de Pfeiffer tipo 1), *POR* (síndrome de Antley-Bixler) e *RAB23* (síndrome de Carpenter) (Johnson & Wilkie, 2011).

A síndrome de Apert

Características gerais

A primeira craniossinostose sindrômica clinicamente descrita foi a síndrome de Apert em 1894 por SW Wheaton; todavia, ele atribuiu erroneamente a causa do fenótipo calvarial à sífilis congênita (Cunningham et al., 2007). Em 1906, o pediatra francês Eugène Charles Apert (1868-1940) fez a revisão literária de oito pacientes e descreveu um nono paciente por ele examinado, para os quais ele propôs que fosse uma única entidade clínica (Cunningham et al., 2007; Lee & Chung, 2010). No trabalho publicado sob o título “*De l'acrocephalosyndactylie*”, ele escreve:

“Eu sugiro o nome acrocefalosindactilia para designar um tipo de teratologia compatível com a vida e fortemente caracterizada pela coexistência das duas seguintes particularidades: Primeiro, um crânio alto e achatado na parte posterior e por vezes nas laterais, apresentando abaulamento do lado oposto a uma faceta exagerada na região superior frontal; segundo, sindactilia das quatro extremidades.”

Somente em 1960, em uma revisão de 39 casos realizada por Blank (Blank, 1960), a acrocefalosindactilia típica, tal como descrita por Eugène Apert, passou a ser denominada síndrome de Apert (Lee & Chung, 2010).

A Síndrome de Apert é, portanto, uma doença congênita caracterizada por craniossinostose, hipoplasia do terço médio da face e sindactilia simétrica das mãos e pés, tendo ao menos os dígitos 2, 3 e 4 envolvidos (Cohen, 1975; OMIM #101200). O crânio, além da sinostose, que é devida à fusão das suturas coronais, apresenta também um defeito calvarial de linha média, que vai desde a glabella até a fontanela posterior, sendo que as suturas sagitais e metópica não se formam (Figura I.4). A megalencefalia verdadeira é característica da síndrome de Apert, com aumento tanto no peso quanto no volume do encéfalo (Cohen & Kreiborg, 1993 a, b, c, d), distinguindo-a de outras craniossinostoses sindrômicas. Além disso, 72% dos pacientes apresentam anormalidades cerebrais (Renier

et al., 1996a; Raybaud & Di Rocco, 2007), como a disfunção neurológica e alterações estruturais do cérebro (principalmente agenesia do corpo caloso, ventriculomegalia e anomalia do septo) que não são dependentes de procedimentos cirúrgicos.

Os pacientes com síndrome de Apert são limitados pela diminuição da amplitude de movimento do ombro, cujo impacto para o indivíduo é de importância igual às anomalias de mão e do pé (Kasser & Upton, 1991; Murnaghan et al., 2007). Acne moderada a severa é outro fenótipo característico da síndrome de Apert, que começa no início da puberdade (Chen et al., 2010). Outras anomalias viscerais podem estar presentes em indivíduos portadores da síndrome de Apert, entre as quais se incluem as malformações cardiovasculares (presentes em 10% dos casos) e genitourinárias (9,6%) e anomalias no sistema respiratório (1,5%) e gastrointestinal (1,5%) (Cohen, 1975; Cohen & Kreiborg, 1993c).

A prevalência da síndrome de Apert é de 1 a cada 65.000 nascidos vivos e esta síndrome representa 4% de todos os casos de craniossinostose (Cohen et al., 1992; Tolarova et al., 1997). O padrão de herança desta síndrome é autossômico dominante e sua razão sexual homem: mulher é de 1:1 (Cohen & Kreiborg, 1991; Tolarova et al., 1997). Apesar de terem sido reportadas 16 casos familiares de síndrome de Apert (Cohen, 2000), a maior parte dos casos é de mutações de novo, sendo que a origem de novas mutações é exclusivamente de origem paterna (Moloney et al., 1996) e a idade paterna está associada à doença (Glaser et al., 2003). Sabe-se que a mutação mais frequente associada a essa Síndrome, p.S252W em FGFR2, gera vantagem seletiva em espermatozônias humanas (Goriely et al., 2005). A raridade de casos familiares é explicada pelo reduzido valor adaptativo genético (fitness) dos indivíduos afetados, ocasionado pelas malformações graves e retardo mental associado em alguns casos, o que diminui a probabilidade desses pacientes encontrarem parceiros e deixarem descendentes (Cohen & Kreiborg, 1991).

A única forma de tratamento para estes pacientes é a intervenção cirúrgica, que consiste em descompressão crânio-orbital, com a reconstrução das suturas coronais (suturectomia) e osteotomia da caixa craniana anterior e das órbitas superiores com remodelamento e avanço (Posnick et al., 1995). No entanto, é observado que após as cirurgias, as suturas rapidamente reossificam, de modo que qualquer operação para craniossinostose durante a infância é meramente um procedimento de atraso da sinostose (Cohen, 2000), ressaltando a importância de prover suturas artificiais que permaneçam abertas durante a fase de crescimento do indivíduo.

Figura 1.5. Características clínicas dos pacientes de síndrome de Apert. (A) e (B) Pacientes de síndrome de Apert com a mutação S252W, apresentando características faciais típicas (A1, A2, B1) e sindactilia das mãos (A3, B2) e dos pés (B3). (C) e (D) Pacientes de síndrome de Apert com a mutação P253R, que apresentam sindactilia mais grave do que os pacientes portadores da mutação S252W (C2). Tomografia computadorizada da paciente D mostra o defeito calvarial de linha média (D2) e a fusão da sutura coronal (D3, seta vermelha). (E) Paciente com mutação atípica em sítio de acceptor de splice (c.1119-2 A>G) de FGFR2, que apresenta características faciais e defeitos de membros característicos da Síndrome de Apert.

Etiologia molecular

Em 1995, Wilkie et al. descobriram duas mutações no gene *FGFR2* (Fibroblast Growth Factor Receptor 2): c.1028C>G (Genebank accession number M87770), resultando em p.S252W, e c.1031C>G (Genebank accession number M87770), resultando em p.P253R. Estas mutações foram confirmadas para a Síndrome de Apert em outros estudos, incluindo um de autoria de nosso grupo (Park et al., 1995b; Passos-Bueno et al., 1998; Tsai et al., 1998; Lajeunie et al., 1999).

Outras mutações em *FGFR2* associadas à Síndrome de Apert também já foram relatadas. Oldridge et al. (1997) e Lajeunie et al. (1999) encontraram uma troca de dois nucleotídeos 755_756CG>TT que resulta em p.S252F (Oldridge et al., 1997; Lajeunie et al., 1999). A raridade desta mutação é justificada por consistir na troca de dois nucleotídeos. Em 1997, nosso grupo encontrou uma mutação em sítio de acceptor de splice (c.1119-2 A>G) em uma paciente com Síndrome de Apert, sendo que esta mutação é geralmente associada à Síndrome de Pfeiffer (Passos-Bueno et al., 1997). Oldridge et al. (1999; 2009) reportaram inserções Alu neste gene (Oldridge et al., 1999; Bochukova et al., 2009).

A fim de aprofundar nos aspectos moleculares da Síndrome de Apert, é necessário entendimento da importância e função da via de sinalização por FGFs.

A via de sinalização por FGFs

Os fatores de crescimento de fibroblasto

Os FGFs de mamíferos são 18 glicoproteínas com pesos moleculares que variam entre 17 e 34 KDa (FGF1-FGF10 e FGF16-FGF23) agrupados em seis subfamílias baseadas na homologia e filogenia (Figura I.5A). Apesar dos FHF (Fibroblast homologous factors: FGF11, FGF12, FGF13 e FGF14) terem sequência de alta homologia estrutural com os FGFs e se ligarem com alta afinidade à heparina, eles não ativam os FGFRs e, portanto, não serão considerados para discussão.

Em comum, os FGFs possuem uma região homóloga de 120 a 130 aminoácidos com 40 a 60% de identidade, dos quais 28 são altamente conservados e 6 são idênticos em todos os FGFs (Givol & Yayon, 1992; Burke et al., 1998), ordenados em 12 folhas-beta antiparalelas ($\beta 1$ - $\beta 12$) flanqueadas com domínios amino e carboxi terminais divergentes (Figura I.5B). Em geral, é a variação na sequência primária das regiões N- e C-terminais que diferenciam a biologia destes ligantes (Nickel, 2005) (Figura I.5C). O loop da região $\beta 1$ - $\beta 2$ e partes da região abrangendo $\beta 10$ e $\beta 12$ correspondem

ao sítio de ligação a heparan sulfato glicosaminoglicanas (HSGAG). Os elementos deste sítio geralmente formam uma superfície contínua e positivamente carregada, contudo, na subfamília dos FGFs endócrinos (FGF19, FGF21 e FGF23), o loop β 1- β 2 e a região β 10- β 12 formam cristas que reduzem estericamente a ligação a HSGAG (Goetz et al., 2007).

A variedade de funções dos FGFs se reflete na sua localização tecidual, expressão temporal e especificidade de ligação aos receptores. Alguns são expressos apenas durante a embriogênese (FGFs 3, 4, 8 e 17), enquanto outros (FGFs 1, 2, 5-7, 9-10, 16, 18-23) podem ser encontrados tanto em tecidos embrionários quanto adultos. Todos os FGFs são encontrados na matriz extracelular. O FGF1 também é conhecido como FGF ácido e o FGF2, como FGF básico, sendo ambos os FGFs mais estudados. O FGF1 é considerado um ligante universal, capaz de se ligar a todos os tipos conhecidos de FGFRs.

Os receptores de fatores de crescimento de fibroblasto

Os FGFs exercem sua ação biológica através de quatro receptores tirosino-quinases transmembrânicos: FGFR1, FGFR2, FGFR3 e FGFR4. Há ainda um quinto FGFR, conhecido como FGFR5 ou FGFR1 (FGFR-like 1), que pode se ligar a FGFs, mas não apresenta domínio tirosino-quinase, e para o qual foi proposto a função de regulador negativo da sinalização FGF (Wiedemann & Trueb, 2000). Os FGFR1-FGFR4 são receptores que consistem em um domínio extracelular, formado por três alças semelhantes à Imunoglobulina (*“immunoglobulin-like domains”*, IgI, IgII e IgIII), cada um mantido por ligações de ponte de dissulfeto; um domínio transmembrânico único de aproximadamente 22 aminoácidos e dois domínios tirosino quinases citoplasmático (Givol & Yayon, 1992; Johnson & Williams, 1993; Spivak-Kroizman et al., 1994) (Figura I.6A). No domínio extracelular, a alça Ig-like III é a mais conservada das três alças (Givol & Yayon, 1992; Johnson & Williams, 1993). A marca registrada dos FGFRs é a presença de uma sequência ácida e rica em serina entre o IgI e o IgII, a caixa ácida, para a qual se acredita que tenha papel de autoinibição do receptor (Wang et al., 1995). O domínio justamembrana contém um sítio de ancoragem para FRS2 e os dois domínios tirosino-quinases são fosforilados na ativação do receptor (Hajihosseini, 2008). O domínio intracelular é o mais conservado entre os FGFRs.

A transcrição de genes que codificam três dos FGFRs (FGFR1, 2 e 3) resulta na expressão de várias isoformas dos receptores devido à ocorrência de processamentos alternativos, o que determina o número de domínios Ig-like (dois ou três domínios) e, mais importante, *splicing* alternativo

C

				$\beta 1$	
[	FGF1	14	E K F N L P P G N Y K K P K L L Y C	31	
[	FGF2	37	G S G A F P P G H F K D P K R L Y C	34	
[	FGF3	34	G G V Y E H L G G A P R R R K L Y C	51	
[	FGF7	55	R S Y D Y M E G G D I R V R R L F C	72	
[	FGF10	68	V R S Y N H L Q G D V R W R K L F S	85	
[	FGF22	31	P R S Y P H L E G D V R W R R L F S	48	
[	FGF4	72	S G A G D Y L L G I K R L R R L Y C	89	
[	FGF5	67	Q S S F Q W S P S G R R T G S L Y C	84	
[	FGF6	74	N W E S G Y L V G I K R Q R R L Y C	91	
[	FGF8	42	L V T D Q L S R R L I R T Y Q L Y S	59	
[	FGF17	43	A M T D Q L S R R Q I R E Y Q L Y S	60	
[	FGF18	43	R A R D D V S R K Q L R L Y Q L Y S	60	
[	FGF9	49	V T D L D H L K G I L R R R Q L Y C	66	
[	FGF16	48	P T D F A H L K G I L R R R Q L Y C	65	
[	FGF20	52	A A Q L A H L H G I L R R R Q L Y C	69	
[	FGF19	33	P H V H Y G W G D P I R L R H L Y T	50	
[	FGF21	35	S S P L L Q F G G Q V R Q R Y L Y T	52	
[	FGF23	27	N A S P L L G S S W G G L I H L Y T	44	

Figura I.6. Os fatores de crescimento de fibroblastos, FGFs. (A) A análise filogenética indica que os 18 genes FGF e 4 genes FHF podem ser organizados em sete subfamílias, contendo dois a quatro membros cada. O comprimento de cada ramo é proporcional à distância evolutiva entre cada gene (retirado de Itoh & Ornitz, 2004). (B) Estrutura tridimensional do FGF2, mostrando as 12 folhas-beta antiparalelas (retirado do banco de dados RCSB Protein Data Bank, ID: 1BAS). (C) O alinhamento das sequências divergentes da região N terminal proximal ao núcleo das folhas-beta dos 18 FGFs, agrupados de acordo com a subfamília. A posição da folha $\beta 1$ do FGF1 está indicada (Retirado de Beenken & Mohammadi, 2009).

dos exons que codificam a região C-terminal do domínio IgIII. Este processamento alternativo aumenta o grau de diversidade molecular, uma vez que expande as propriedades de ligação do receptor a diferentes FGFs (Givol & Yayon, 1992; Johnson & Williams, 1993; Ornitz et al., 1996). O *splicing* alternativo é tecido-específico e produz a isoforma “c” (ex.: FGFR1c, FGFR2c e FGFR3c), cuja alça Ig-III é codificada pelos exons IIIa e IIIc (exons 8 e 10 em camundongos; 9 e 11 em humanos), a mais abundante e expressa em tecidos de origem mesenquimal; e a isoforma “b” (ex.: FGFR1b, FGFR2b e FGFR3b), cuja alça Ig-III é codificada pelos exons IIIa e IIIb (exons 8 e 9 em camundongos; 9 e 10 em humanos) menos frequente e expressa em tecidos de origem epitelial (Miki et al., 1992; Orr-Urtreger et al., 1993; Chellaiah et al., 1994; Naski & Ornitz, 1998) (Figura I.6B). As propriedades de ligação de cada isoforma são bastante específicas (Tabela I. 2).

Figura I.7. Os receptores de fator de crescimento de fibroblasto, FGFRs. (A) Esquema da estrutura de um FGFR (B) Splicing alternativo do domínio IgIII, responsável pela especificidade do ligante. A primeira metade do Ig III é codificado por um exon invariável (IIIa), a segunda metade pode ser codificado pelo exon IIIb ou pelo IIIc, que é seguido pelo domínio tranmembrânico, codificado pelo exon TM Tecidos epiteliais expressam predominantemente a isoforma IIIb e tecidos mesenquimais, a IIIc . FGFR4 é expresso como uma isoforma única, paráloga à FGFR-IIIc.

Tabela I. 2. Especificidade de ligantes de isoformas de FGFRs (extraído do banco de dados Reactome)

Gene	Localização	Proteína	Ligantes específicos
<i>FGFR1</i>	8p12	FGFR1b FGFR1c	FGF 1-3, 10 e 22 FGF1, 2, 4-6, 8, 9, 17, 20 e 23
<i>FGFR2</i>	10q26.12	FGFR2b FGFR2c	FGF1, 3, 7, 10 e 22 FGF1, 2, 4-6, 8, 9, 16-18, 20 e 23
<i>FGFR3</i>	4p16.3	FGFR3b FGFR3c	FGF1, 8, 9, 17, 18 e 20 FGF1, 2, 4, 5, 8, 9, 16-18, 20 e 23
<i>FGFR4</i>	5q35.2	FGFR4	FGF1-9, 17- 20 e 23

A ativação de FGFRs por FGFs

A unidade funcional de FGF-FGFR consiste de dois complexos FGF-FGFR-HSGAG (na razão de 1:1:1) justapostos em um dímero simétrico (Schlessinger et al., 2000). Cada ligante no dímero se liga ao receptor na alça entre os domínios IgII e IgIII, enquanto que os receptores se contatam diretamente na região da base do IgII. Cada ligante se liga ao segundo receptor do dímero através do domínio IgII (Ibrahimi et al., 2005) (Figura I.8B). O HSGAG presente na matriz extracelular se liga a um “cânion” básico formado na porção distal à membrana do dímero simétrico, fortalecendo e estabilizando o dímero, por se ligar simultaneamente ao ligante e ao receptor (Figura I.8B). Além disso, o HSGAG estabiliza os FGFs contra a degradação, serve como reservatório de armazenamento do ligante e determina o raio de difusão do ligante (Hacker et al., 2005).

Os monômeros de FGFR adotam uma configuração de autoinibição quando o IgI e a caixa ácida ocupam o sítio de ligação intramolecular, na alça entre o IgII e o IgIII. Considera-se que este estado “fechado” encontra-se em equilíbrio com o estado “aberto”, no qual os sítios de ligação se encontram vazios e preparados para interagir com FGF e HSGAG, possibilitando a ativação do FGFR (Lemmon & Schlessinger, 2010) (Figura I.8A). Tirosinas no circuito de ativação de FGFR participam de um conjunto único de contatos intramoleculares que estabilizam a conformação inativa da quinase, ao passo que induzem à cis autoinibição do domínio tirosino quinase pela oclusão do sítio de ligação de proteínas-substrato, mas não do sítio de ligação ao ATP.

Quando o FGF induz a dimerização de seu receptor, a transfosforilação das tirosinas rompe a configuração cis-autoinibitória de forma que a alça de ativação e a hélice α C da quinase N-terminal adotam uma configuração ativa característica (Lemmon & Schlessinger, 2010) (Figura I.8B). Acredita-se que a ativação de FGFR ocorra em três fases. Estudos com FGFR1 mostram que a autofosforilação de Y653 aumenta a atividade da quinase de 10 a 50 vezes e é o evento de autofosforilação chave durante a primeira fase (Furdui et al., 2006) (Figura I.8B). Em seguida, eventos de autofosforilação na segunda fase ocorrem em uma ordem precisa: Y583, que fica em um loop extra dentro do domínio tirosino-quinase (a inserção quinase), é trans-autofosforilada primeiro; Y463 na região justamembrana é a próxima a ser fosforilada, seguida por Y585 na inserção quinase. Estas três tirosinas da segunda fase são sítios de ancoragem de domínio SH2/PTB cuja fosforilação promove o recrutamento de moléculas de sinalização a jusante, em vez de aumentar a atividade da quinase (Figura I.8B e C). Após esta segunda fase, um evento de autofosforilação ainda ocorre no circuito de ativação de FGFR1 em Y654, aumentando a atividade da quinase em mais 10 vezes (Furdui et al., 2006), alcançando 100-500 vezes acima da atividade quinase dos níveis basais. Esta modificação do Y654 representa a “terceira

fase” da autofosforilação que estimula ao máximo o domínio quinase de FGFR1 para a fosforilação de alvos a jusante, tais como a fosfolipase C- γ (PLC γ) e do substrato de receptor de FGF -2 (FRS2) (Figura I.8B e C). A fosforilação da Y654 não é necessária para eventos de autofosforilação do receptor na segunda fase que direcionam para a montagem de moléculas de sinalização no receptor ativado (Lemmon & Schlessinger, 2010).

Figura I.8. Ativação subsequente sinalização intracelular dos FGFRs (A) O receptor FGFR no estado inativo, na qual os monômeros em estado aberto (esquerda) e fechado (direita) se encontram em equilíbrio. (B) Ativado, os monômeros de FGFRs formam dímeros e desencadeiam uma cascata de trans-autofosforilação em três fases (1, 2 e 3). (C) Cascata de sinalização intracelular desencadeada pela ativação dos FGFRs e as possíveis consequências de cada via (adaptado do banco de dados do software Ingenuity).

A sinalização intracelular em resposta à ativação de FGFRs

A primeira resposta a autofosforilação de FGFRs é o recrutamento e ativação de uma série de moléculas de sinalização a jusante. Essas moléculas contêm domínios SH2 ou PTB, que se ligam especificamente a fosfotirosina (pY) (Schlessinger & Lemmon, 2003; Pawson, 2004). Elas podem ser recrutados diretamente para as pY do receptor ou indiretamente pela ligação a proteínas de ancoragem, como o FRS2, que são fosforilados pelo FGFR (Schlessinger et al., 2000) (Figura I.8C). As proteínas de ancoragem geralmente contêm um sítio de localização na membrana sua porção N-terminal, seguido por uma série de sítios de fosforilação de tirosina que servem como sítios de ligação para um repertório distinto de proteínas de sinalização a jusante. Assim como em outros receptores tirosino-quinases, com a presença de múltiplas pY e o envolvimento de várias proteínas de ancoragem, os FGFRs ativados recrutam e influenciam uma grande variedade de moléculas de sinalização. Portanto, um receptor ativado pode ser considerado como um nó em uma complexa rede de sinalização, que transmite informações a partir do exterior para o interior da célula.

A comunicação entre as moléculas de sinalização em redes influenciadas por receptores tirosino-quinase se dá através de uma ampla gama de módulos de interação (Seet et al., 2006). Alguns módulos (como o SH2 e o PTB) se ligam diretamente ao receptor (interações proximais), enquanto outras interações são espacialmente e temporalmente mais distais. As interações proximais exigem a modificação do receptor. O SH2 e PTB se vinculam apenas ao receptor tirosina-fosforilado e, posteriormente, atrelam a autofosforilação do FGFR ao início de outros eventos da rede de sinalização (Schlessinger & Lemmon, 2003; Pawson, 2004). Vários módulos de ligação a ubiquitina também se ligam diretamente ao receptor ubiquitinado (Hurley et al., 2006). A ubiquitinação, que frequentemente depende da ativação do receptor, sinaliza a internalização e degradação do receptor para atenuar a sinalização do fator de crescimento (Schlessinger et al., 2000; Kirkin & Dikic, 2007), criando um mecanismo de retroalimentação negativa importante (Hunter, 2007). Os módulos de interações distais se localizam frequentemente ao lado de domínios SH2 em proteínas de múltiplos domínios e podem ser classificados em duas categorias: os domínios SH3, WW, e PDZ são exemplos de módulos de interação proteína-proteína, enquanto o PH, PX, C1, C2, e os domínios FYVE são mais conhecidos como módulos de interação a fosfolipídios.

Embora estes domínios conduzam e especifiquem a formação dos principais complexos de sinalização (Seet et al., 2006), eles não mostram seletividade de ligação ou afinidade suficiente para explicar por conta própria a especificidade necessária para a formação do complexo de sinalização (Ladbury & Arold, 2000). O que deve estabelecer esta especificidade é a multivalência, na qual vários domínios em uma única proteína de sinalização, em resposta a estímulos diversos, cooperam entre si

para direcionar a sinalização (Pawson, 2004; Seet et al., 2006), seja para ativação de vias de sinalização intracelular da fosfolipase C (PLC), da proteína quinase C (PKC), da Ras ativada por mitógeno proteína quinase (MAPK) ou do fosfatidilinositol 3-quinase (PI3K)-Akt (PI3K/Akt) (Mohammadi et al., 2005; Thisse & Thisse, 2005; Miraoui & Marie, 2010) (Figura I.8C).

As principais vias de sinalização ativadas por FGFRs se encontram descritas abaixo e na Figura I.8C e são compreendidas em parte, principalmente nas porções iniciais. Entretanto, é perceptível que grande parte do funcionamento e do controle dessas vias não é entendida.

Via da PLC e via da PKC

Ocorrem através da via do fosfoinositol, que gera mensageiros secundários. O domínio SH2 do pLCY hidrolisa o fosfatidilinositol-(4,5)-bisfosfato (PIP2) em diacilglicerol (DAG) e inositol trifosfato (IP3). O diacilglicerol ativa proteínas da família das proteínas kinases C (as quais, quando se encontram inativas, são proteínas solúveis e citossólicas), que passam a regular a atividade gênica. O IP3 estimula a liberação de íons Ca^{2+} , que se ligam a calmodulina e ativam proteínas kinases dependentes de calmodulina. Esta via pode levar a fosforilação de vários fatores de transcrição gênica e induzir ou reprimir a síntese de alguns RNAs mensageiros. É importante para a manutenção do metabolismo e do crescimento celular (Yamaguchi et al., 1995).

Via Ras-MAPK

A via mais comumente estimulada por FGFRs é a de Ras, por desfosforilação de GTP em GDP. Neste processo, Grb2 se liga a pY através do seu módulo SH2 e ao Sos (guanine nucleotide exchange factor) através do módulo SH3, recrutando Sos para a membrana plasmática, onde este estimula Ras ao catalisar a desfosforilação de GDP em GTP. O Ras ativado (ligado ao GTP) agora interage com Raf (uma serina quinase) que, por sua vez, estimula a MAP quinase quinase (MAPKK, também conhecidas como MEKS), fosforilando resíduo de serina. A MEK fosforila e ativa MAPK (ERK), que cruza a carioteca em direção ao núcleo, onde fosforila e ativa vários fatores de transcrição, como o CREB, que, por sua vez, ativam vários genes em resposta ao FGF. Esta cascata é altamente conservada evolutivamente e tem um papel importante no controle da diferenciação e proliferação celular (Marshall, 1995; Park et al., 1995a; Kauhara et al., 1997).

Via da PI3K-Akt

Fosfoinositídeo 3-quinase de classe Ia são heterodímeros que consistem de uma subunidade reguladora p85 e uma subunidade p110 catalítica. Os domínios SH2 do p85 se ligam a resíduos de pY do FGFR ativado. Esta ligação recruta o heterodímero p85-P110 para a membrana plasmática, onde o seu substrato, PIP2, reside e alivia a inibição basal de p110 pelo p85 (Yu et al., 1998). O acúmulo de PIP3 na membrana da célula leva à co-localização de proteínas de sinalização com domínios PH, ativando estas proteínas e propagando a sinalização PI3K. O Akt e o fosfatidilinositol dependente da proteína quinase 1 (PDK1) se ligam diretamente a PIP3 e são, assim, recrutados para a membrana plasmática. O Akt fosforila várias proteínas celulares, incluindo GSK3 α , GSK3 β , FOXO, MDM2, BIM e BAD, e está relacionado a sobrevivência da célula e entrada no ciclo celular (Cantley, 2002).

Funções biológicas da via de FGFs

A fim de assegurar a morfogênese apropriada durante o desenvolvimento de organismos multicelulares, muitas vias de sinalização são ativadas de modo altamente coordenado através de moléculas sinalizadoras secretadas, tais como os FGFs. Nos vertebrados, a sinalização por estas moléculas evoluiu, tornando-se bastante complexa, o que reflete na amplitude de funções fisiológicas que são controladas por estes fatores não só durante a embriogênese, mas também na homeostase tecidual no adulto. Desde sua descoberta em 1973 (Armelin, 1973), estas moléculas e seus receptores já foram implicados tanto em processos biológicos mitogênicos, regulatórios, morfológicos quanto em processos endócrinos (Beenken & Mohammadi, 2009). Isso é evidenciado na Tabela I. 3, que mostra os estudos que investigaram o papel da maioria dos FGFs conhecidos de mamíferos por meio de inativação gênica (nockout por recombinação homóloga) em modelos murinos. A variabilidade fenotípica observada nestes estudos ressalta as diferenças na expressão temporal e espacial desses genes e no desencadeamento de diferentes vias de sinalização pelos mesmos, ressaltando a relevância da sinalização por FGFs em uma diversidade vasta de processos fisiológicos.

A sinalização por FGFs na síndrome de Apert

Como já mencionado, mutações no gene *FGFR2* causam a Síndrome de Apert. A mutação p.S252W é a mais frequente, corresponde de 62 a 71% dos casos e leva a um quadro clínico cranio-

Tabela 1. 3. Resultados de estudos com modelos de camundongos nocaute para diferentes Fgfs.

Gene	Sobrevivência	Fenótipo	Referência
<i>Fgf1</i>	Viável	Nenhuma anomalia encontrada	(Miller et al., 2000)
<i>Fgf2</i>	Viável	Defeitos neuronais, ósseos e epidérmicos, além de hipotensão. Inibição de formação óssea.	(Dono et al., 1998, Montero et al., 2000)
<i>Fgf3</i>	Viável	Malformações no ouvido interno e defeitos no desenvolvimento do rabo	(Mansour et al., 1993)
<i>Fgf4</i>	Letal (morte no estágio E5.5)	Proliferação prejudicada de células da massa interna	(Feldman et al., 1995)
<i>Fgf5</i>	Viável	Pelos anormalmente longos	(Hebert et al., 1994)
<i>Fgf6</i>	Viável	Prejuízo na regeneração dos músculos esqueléticos	(Floss et al., 1997)
<i>Fgf7</i>	Viável	Anomalias nos folículos pilosos e deficiência renal	(Guo et al., 1996)
<i>Fgf8</i>	Letal (morte no estágio E8.5)	Defeitos na gastrulação e no desenvolvimento encefálico, cardíaco e craniofacial.	(Meyers et al., 1998, Sun et al., 1999)
<i>Fgf9</i>	Letal (morte pós-natal)	Hipoplasia pulmonar e reversão de sexo	(Colvin et al., 2001a, Colvin et al., 2001b)
<i>Fgf10</i>	Letal (morte pós-natal)	Ausência dos pulmões e dos membros, tanto anteriores quanto posteriores.	(Min et al., 1998, Sekine et al., 1999)
<i>Fgf15</i> (ortólogo do <i>FGF19 humano</i>)	Letal (morte pós-natal)	Anomalias cardiovasculares que afetam principalmente os ventrículos	(Vincentz et al., 2005)
<i>Fgf17</i>	Viável	Defeitos no desenvolvimento do Mesencéfalo e do Rombencéfalo	(Xu et al., 2000)
<i>Fgf18</i>	Letal (morte pós-natal)	Atraso na ossificação, aumento na proliferação dos condrócitos e diminuição dos espaços alveolares nos pulmões.	(Liu et al., 2002, Ohbayashi et al., 2002, Usui et al., 2004)
<i>Fgf20</i>	Viável	Comprometimento do desenvolvimento do ouvido interno	Ornitz, 2009
<i>Fgf21</i>	Viável	Hipertrofia e diminuição da lipólise nos adipócitos	(Itoh & Ornitz, 2008)
<i>Fgf22</i>	Viável	Diferenciação sináptica prejudicada e atraso no ganho de peso	(Terauchi et al., 2010)
<i>Fgf23</i>	Viável	Hiperfosfatemia, hipoglicemia, densidade óssea reduzida e infertilidade.	(Shimada et al., 2004)

facial mais grave, enquanto a mutação p.P253R, a segunda mais frequente, corresponde de 26 a 37% dos casos (Park et al., 1995b; Moloney et al., 1996; Slaney et al., 1996; Oldridge et al., 1999) e está associada a uma forma mais grave de sindactilia das mãos e dos pés (Slaney et al., 1996; von Gernet et al., 2000). A amostra de pacientes de Apert atendidos pelo nosso grupo possui a frequência de mutações similar à da literatura, de 64% (p.S252W) e 26% (p.P253R), em uma amostra total de 72 pacientes até o presente momento. Essas mutações estão localizadas na região de ligação entre as alças IgII e III de FGFR2, encontradas em ambas as isoformas (FGFR2b e FGFR2c).

A isoforma selvagem de FGFR2b se liga a FGF1, 3, 7, 10 e 22; e a isoforma selvagem de FGFR2c a FGF1, 2, 4, 5, 6,8, 9, 16, 17, 18, 20 e 23 (Ornitz et al., 1996; Xu et al., 2000; Umemori et al., 2004) (Tabela I. 2). A isoforma FGFR2c portadora da mutação p.S252W, quando comparada à proteína selvagem e à isoforma FGFR2c portando a mutação p.P253R, apresenta aumento de afinidade pela maior parte dos FGFs (Ibrahimi et al., 2004). Ibrahimi e colaboradores demonstraram que estas mutações aumentam a afinidade de ligação dos receptores FGFR2b e FGFR2c por quase todos os FGFs e levam à perda de especificidade aos ligantes, de forma que os receptores se liguem indiscriminadamente a vários FGFs e podem levar a ativação autócrina da sinalização das diferentes isoformas de FGFR2 por ligantes que são expressos pelo mesmo tecido (Ibrahimi et al., 2004).

A associação molecular entre o FGFR2 e a formação óssea tem sido alvo de muitos estudos. Atualmente a relação FGF2-FGFR2 e o fator de transcrição RUNX2, importante para o início da osteogênese, está bem caracterizada. Em osteoblastos, a via de sinalização mediada por FGF2/FGFR2 que leva à ativação de RUNX2, é a das proteínas quinase C, sendo que a isoforma envolvida é PKC α (Kim et al., 2003). Também foi demonstrada a relação funcional entre TWIST1, FGFR2 e RUNX2 em um modelo de estudo de crescimento craniano normal e na Síndrome de Saethre-Chotzen (craniossinostose síndrômica de suturas coronais causada por mutações em *TWIST1*) em osteoblastos (Guenou et al., 2005). Foi verificado que a proteína TWIST1 se liga a uma região específica do promotor do gene FGFR2 *in vivo* e que, em células *TWIST1* mutantes, a ligação de RUNX2 ao promotor de FGFR2 está diminuída. Sabe-se também que as proteínas TWIST (TWIST 1 e 2) inibem o funcionamento de RUNX2 durante o desenvolvimento esquelético de camundongos pela interação de seu domínio Twist box com o domínio de ligação ao DNA de Runx2 (Bialek et al., 2004).

Modelos animais para a Síndrome de Apert

Modelos animais têm sido utilizados no estudo do papel *FGFR2* na Síndrome de Apert ou de outras craniossinostoses causadas por mutações neste gene. Em 2001, Hajihosseini e colaboradores

descreveram a supressão completa do exon 9 (IIIc) do *Fgfr2*, incluindo porções significativas dos íntrons 8 e 9, em camundongos. A sua supressão causou uma mutação letal dominante com a alteração no splicing e induziu a expressão ectópica de *Fgfr2b*, levando, em animais heterozigotos, a graves anomalias viscerais e esqueléticas (Hajihosseini et al., 2001) comparáveis as vistas em pacientes de síndrome de Apert e de Pfeiffer. Quando a expressão de *Fgf10* foi inibida nestes animais, houve um resgate dos defeitos viscerais e de esqueleto, intermediado por ERK e p38 MAPK (Hajihosseini et al., 2009).

Em 2002, o grupo de Eswarakumar também criou camundongos nocaute para a isoforma FGFR2c, porém através da inserção de um códon de parada no exon 9 (IIIc) (Eswarakumar et al., 2002). Os camundongos *Fgfr2 IIIc^{-/-}* apresentaram atraso no início da ossificação por todo o esqueleto, seguido de fusão prematura dos ossos cranianos, incluindo as suturas coronais e as sincondroses da base do crânio, e menor crescimento dos ossos longos. Esses defeitos estavam associados à perda prematura de capacidade proliferativa em células osteoprogenitoras na sutura coronal e em condrócitos nas placas de crescimento de ossos endocondrais. De modo similar, a inativação condicional de *Fgfr2* mostrou que a ativação de *Fgfr2* é essencial para a proliferação de osteoblastos, mas não na diferenciação (Yu et al., 2003).

Existem atualmente três modelos murinos com a mutação p.S252W em *Fgfr2* produzidos independentemente (Chen et al., 2003; Wang et al., 2005; Holmes et al., 2009) com resultados contraditórios, sumarizados na Tabela I. 4.

No estudo de 2003, Chen e colaboradores inseriram a mutação através de um vetor ploxneo, na qual a sequência da neomicina se localizava a montante do exon 7 (IIIa), onde fica a mutação. Estes animais apresentaram sinostose coronal sem sindactilia. A característica fenotípica mais importante que observaram foi a formação óssea diminuída e aumento de apoptose nas suturas coronais, sugerindo um possível papel da apoptose como um mecanismo celular relevante para algumas formas de

Tabela I. 4. Principais diferenças fenotípicas observadas nos diferentes modelos murinos para a Síndrome de Apert, mutação p.S252W em *Fgfr2*.

	Chen et al., 2003	Wang et al., 2005	Holmes et al., 2009
Fusão da sutura coronal	Pós natal	Entre E18.5 e P1	Entre E15.5 e E16.5
Mortalidade	Morte 20 dias pós natal ou até vida adulta	Entre 24 a 36 hs pós natal	Alguns dias após nascimento
Proliferação na sutura	Sem diferença (E16.5 a P8)	Aumento em 2 vezes após E18.5	Aumento em E12.5 e diminuição em E15.5
Apoptose na sutura	Aumento em E18.5 até P8	Sem diferença (E16.5 a P1)	Aumentada em E16.5, mas secundária ao fechamento da sutura
Expressão de marcadores de osteoblastos	Sem diferença (E18.5 a P18)	Aumento em E18.5	Aumento em E15.5

craniossinostoses (Tabela I. 4) (Chen et al., 2003). Utilizando este modelo, Shukla e colaboradores realizaram o tratamento dos camundongos mutantes com U0126, inibidor de MEK1/2, e observaram uma inibição significativa da craniossinostose, implicando a ativação de ERK na patofisiologia da síndrome de Apert (Shukla et al., 2007).

Em 2005, Wang e colaboradores inseriram a mutação p.S252W em camundongos C57BL/6J utilizando um construto na qual o cassete de neomicina se localizava a jusante do exon 7 (Wang et al., 2005). Os resultados destes autores sugerem que a sinostose das suturas coronais pode ser devida tanto a um maior crescimento por proliferação e diferenciação, quanto a um menor crescimento com apoptose através da sutura (Tabela I. 4). Análise tridimensional do encéfalo destes animais em trabalho posterior sugere que as anomalias no sistema nervoso central são primárias, não secundárias ao fenótipo craniano (Aldridge et al., 2010).

Por último, em 2009, Holmes e colaboradores utilizaram a mesma construção de Chen e colaboradores (Chen et al., 2003) em camundongos Swiss-Webster e observaram um leve aumento na proliferação das células osteoprogenitoras, mas a apoptose é insignificante. Durante a fusão das suturas ocorre diminuição significativa da proliferação destas células (Tabela I. 4). Holmes sugere que os principais determinantes da craniossinostose induzida por FGFR2 é a incapacidade de responder aos sinais de que iria interromper o recrutamento ou a promoção de células osteoprogenitoras nos locais onde as suturas devem normalmente formar (Tabela I. 4).

O grupo que criou o primeiro modelo murino Apert com a mutação p.S252W também desenvolveu o modelo murino Apert com a mutação p.P253R, empregando o mesmo sistema (Yin et al., 2008). A análise do crânio e dos ossos longos mostrou a fusão prematura da sutura coronal, encurtamento da base do crânio e placas de crescimento dos ossos longos e o tratamento *in vitro* da calvaria com PD98059, inibidor de Erk 1/2, aliviou parcialmente a sinostose, mais uma vez enfatizando a importância da via de ERK na síndrome de Apert (Yin et al., 2008).

Respostas celulares alteradas

Outra forma de delinear as perturbações na sinalização por FGF na síndrome de Apert é através da resposta celular *in vitro*, o que permite análise bioquímica mais refinada dos efeitos de mutações em *FGFR2* do que nos modelos animais.

Estudos em osteoblastos

Considerando que o principal tecido afetado na síndrome de Apert é o tecido ósseo, é natural que a maioria dos trabalhos a respeito na literatura procure por características aberrantes em osteoblastos portadores de mutações desta síndrome. Entretanto, como está descrito a seguir, os dados a este respeito são contraditórios.

A transfecção de osteoblastos calvariais murinos primários com *FGFR2* com a mutação p.S252W inibiu a diferenciação e induziu a apoptose (Mansukhani et al., 2000). Por sua vez, a transfecção de osteoblastos calvariais de galinha com *FGFR2* com a mutação p.P253R mostrou fraco efeito mitogênico e não alterou a mineralização durante a diferenciação osteogênica (Ratisoonorn et al., 2003). Osteoblastos isolados de ossos longos do modelo murino de Wang et al. (Wang et al., 2005) apresentaram aumento da proliferação e diferenciação, bem como respostas alteradas dessas funções celulares na presença de FGF2 ou FGF10 (Yang et al., 2008). Como neste último estudo, células mesenquimais C3H10T1/2 transfectadas com *FGFR2*^{S252W} também apresentaram aumento na proliferação e na diferenciação osteogênica, que foram diminuídas quando tratadas com inibidor da via ERK1/2 e da via da PKC, respectivamente (Miraoui et al., 2009).

Os primeiros estudos com células extraídas de pacientes de síndrome de Apert com a mutação p.S252W, no caso, pré-osteoblastos calvariais imortalizados, foram realizados pelo grupo do laboratório de Pierre J. Marie. A mutação não alterou a proliferação em condições basais nem em resposta a FGF2, apesar de aumentar a expressão de marcadores de diferenciação osteogênica (Lomri et al., 1998). A imunohistoquímica dos tecidos de onde estas células eram provenientes confirmou estes resultados e mostrou menor marcação de *FGFR2*, apesar dos níveis de mRNA de *FGFR2* nas células não estarem alterados (Lemonnier et al., 2001a). Além disso, estes pré-osteoblastos sofriam mais apoptose por meio da ativação da PKC, aumentando os níveis de caspases e de fragmentação do DNA (Lemonnier et al., 2001b). A via da PKC também foi correlacionada com o maior potencial osteogênico destas células por induzir o aumento de N-caderina (Lemonnier et al., 2001b). Estudo da expressão gênica usando membrana de filtro de nylon com spots de fragmentos de cDNA amplificados por PCR representativos de 588 genes apontou *PKCA*, *IL1A* e *RHOA* como genes com expressão significativamente aumentada nas células mutantes (Lomri et al., 2001). O tratamento dos pré-osteoblastos com inibidor de PKC, inibidor de p38 MAPK ou inibidor de MEKK diminuiu a expressão de *IL1A* e *RHOA* nas células Apert (Lomri et al., 2001).

Os osteoblastos de ossos dos dedos de dois pacientes Apert com a mutação p.S252W apresentaram maior diferenciação osteogênica (Tanimoto et al., 2004). Por sua vez, osteoblastos da sutura coronal de um paciente Apert com a mutação p.P253R apresentou menor taxa de proliferação e maior potencial osteogênico; tratados com FGF2, a proliferação foi estimulada e a diferenciação, inibida (Fragale et al., 1999). Em estudo também com osteoblastos da sutura coronal de três pacientes com a mutação p.P253R, foi confirmada a menor proliferação e mostrou expressão diferencial de *RUNX2* e aumento na expressão de genes de proteínas constitutivas da matrix extracelular, além da diminuição na expressão de metaloproteinases (Baroni et al., 2005).

A análise global de expressão gênica em osteoblastos calvariais de apenas um paciente Apert com mutação p.S252W comparado a osteoblastos de um indivíduo sem craniossinostose assinalou *EGFR* e *PDGFRA* como genes com expressão aumentada em presença da mutação em *FGFR2*, confirmado por imunohistoquímica (Miraoui et al., 2010). A inibição farmacológica dos receptores EGFR e PDGFR reduziu a diferenciação osteogênica via atividade PKCa dependente, apontando para uma possível interação entre vias de diferentes receptores tirosino-quinases (Miraoui et al., 2010).

Estudos em outros tipos celulares

A importância do periósteo da dura-máter para o fechamento adequado da sutura já foi evidenciada em vários estudos (Cohen & Kreiborg, 1993b; Lenton et al., 2005; Rice & Rice, 2008). O periósteo, apesar de poucos estudos, também parece contribuir para este processo. Considerando-se estes dados e a estrutura complexa da sutura, é muito pouco provável que a fusão prematura da sutura e o processo de ressinostose pós-cirúrgica sejam produtos apenas de alterações em um único tipo celular como os osteoblastos, mas que mais provavelmente eles sejam resultado do fenótipo celular alterado originado de perturbações na sinalização e na interação entre diferentes tecidos e células do complexo das suturas. Portanto, é relevante para compreender a patofisiologia da craniossinostose na síndrome de Apert que se olhe também para outros tipos celulares e outros tecidos.

Os fibroblastos de periósteo portadores da mutação p.P253R apresentaram expressão aumentada de proteoglicanas envolvidas na fibrilogênese de colágeno que, no entanto, diminuía perante a administração de FGF2 (Lilli et al., 2007). Os autores sugerem que as alterações na matriz poderiam influenciar na ligação de FGFs ao FGFR2, apesar de eles não terem investigado isso.

Em estudo publicado pelo nosso grupo foi verificado que fibroblastos de periósteo de pacientes com a mutação p.S252W apresentavam maior potencial osteogênico e a análise do transcriptoma de fibroblastos de 7 pacientes e 7 controles identificou 263 genes diferencialmente expressos, muitos dos quais estavam relacionados a proliferação celular, adesão celular e organização da matriz extracelular, além de componentes das vias PI3K e MAPK (Fanganiello et al., 2007).

A fim de avaliar a influência da dura-máter sobre os osteoblastos da sutura, Ang e colaboradores utilizaram o sistema de co-cultura, no qual células da dura de camundongos são cultivados sobre membranas com poros que permitam apenas a passagem de moléculas sinalizadoras para a placa abaixo, onde os osteoblastos murinos são cultivados. As células da dura-máter transfectadas com FGFR2^{P253R} acentuaram a diferenciação óssea de osteoblastos sem mutação em *Fgfr2*, evidenciando a importância da mutação Apert na dura-máter no processo de osteogênese de tecidos adjacentes (Ang et al., 2010).

Questões não respondidas

O único tratamento disponível para estes pacientes é a correção cirúrgica, que por sua vez é considerada como apenas um atraso no processo de sinostose. Assim sendo, uma melhor compreensão do processo de regeneração óssea nesses pacientes poderá resultar em benefícios para melhores condutas terapêuticas destes.

Como detalhamos previamente, o complexo das suturas permitem regular o balanço entre proliferação e diferenciação de precursores osteogênicos (Slater et al., 2008). Notavelmente, muitos estudos têm apontado a importância do periósteo na formação óssea do crânio. O periósteo contribui não só para o crescimento normal do osso, mas também para a consolidação e regeneração óssea (Ito et al., 2001, Orwoll, 2006) e é altamente celular, contendo de células-tronco, fibroblastos, células osteoprogenitoras diferenciadas até osteoblastos (Squier et al., 1990, Allen et al., 2004). Em ossos longos, o periósteo é uma importante fonte de células-tronco esqueléticas/progenitoras durante a reparação óssea (Colnot, 2009). No entanto, como os diferentes tipos celulares do periósteo da calvária interagem e como esse tecido atua em uma situação patológica, como a alteração de sinalização FGF levando a craniossinostose, ainda é desconhecida.

A maior parte dos estudos envolvendo as consequências celulares e moleculares de mutações associadas não só à Síndrome de Apert, mas às craniossinostoses em geral, focaram em osteoblastos. Entretanto, como mencionado anteriormente, os dados neste aspecto têm se mostrado contraditórios. Além disso, estes resultados podem não ser apropriados para responder às questões relacionadas não só ao processo de craniossinostose, mas principalmente o de ressinostose que ocorre nesses pacientes após a intervenção cirúrgica, uma vez que não leva em consideração a interação entre diferentes células que podem regular esse fenótipo.

Portanto, um melhor entendimento do efeito das mutações da Síndrome de Apert sobre células sinalizadoras no periósteo, como as células-tronco e fibroblastos, poderá delinear melhor a patofisiologia desta síndrome.

Objetivos

Se o periósteo contribui para a fusão, prematura e pós-cirúrgica, das suturas coronais na Síndrome de Apert, não só como fonte moléculas de sinalização, mas também de células osteoprogenitoras, então estas células têm funções celulares como proliferação, migração e diferenciação anômalas em resposta a vias de sinalização intracelulares alteradas. Para testar esta hipótese, nós traçamos dois objetivos principais:

- I) Verificar se a mutação S252W, a mais frequente entre os pacientes com Síndrome de Apert, tem um efeito funcional/celular semelhante em duas diferentes potenciais células osteoprogenitoras: fibroblasto e células-tronco mesenquimais;
- II) Verificar se diferentes ligantes a FGFR2, os FGFs, atuam diferentemente nas funções destas mesmas células com mutação S252W.

Para respondermos o primeiro objetivo, desenvolvemos os seguintes objetivos específicos:

- I.a) Verificar o efeito da mutação S252W na proliferação, migração em fibroblastos e células-tronco mesenquimais (MSCs) provenientes do periósteo de suturas coronais;
- I.b) Corroborar que fibroblastos com a mutação S252W têm maior potencial osteogênico (resultado inicialmente publicado pelo nosso grupo (Fanganiello et al., 2007) e identificar possíveis vias de sinalização envolvidas neste processo;
- I.c) Verificar se a mutação S252W interfere na indução osteogênica de MSCs provenientes de periósteo.

Para respondermos a segunda questão, os seguintes objetivos específicos foram desenvolvidos:

- II.a) Verificar se diferentes FGFs estão associados a redes específicas de sinalização celular em fibroblastos com mutação S252W;
- II.b) Tentar estabelecer outras vias importantes para a Síndrome de Apert através do estudo das mutações raras.

II. FGFR2 mutation confers a more drastic gain of function in fibroblasts than in mesenchymal stem cells

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Abstract

Fibroblast growth factor signaling is implicated in the control of several processes including cell proliferation and differentiation, from early development to adult organism homeostasis. Gain-of-function mutations in FGFR2 are causative of several congenital skeletal disorders, including Apert Syndrome (AS). Using human mesenchymal stem cells (MSC) harboring AS mutation S252W obtained from the periosteum, we evaluated the roles of FGF signaling in proliferation, migration and osteogenic differentiation and compared to fibroblasts obtained from the same tissue of these individuals. The growth curves showed opposite effects of the same mutation in these different cell types: while S252W MSC proliferate less, S252W fibroblasts grow much faster. In low FBS medium, enhanced migration was marked in S252W fibroblasts but not in S252W MSCs. FGFR2^{S252W} induces increased osteogenic differentiation in both studied cell types, though the difference in comparison to WT cells was more accentuated in AS fibroblasts, which was reversed by the inhibition of JNK. We also demonstrated that S252W fibroblasts signaling augment osteogenic differentiation of periosteal MSCs but surprisingly not of MSCs from other tissue. All in all, MSCs and fibroblasts responded differently to the pathogenic effects of the mutation. While the excessive FGF signaling due to FGFR2^{S252W} had more drastic effect on fibroblasts, it also granted the fibroblasts to be more influential over MSCs than contrariwise. Moreover, AS fibroblasts signals only to MSC cells of the same origin, pointing out that MSCs from different niches might have a specific gene imprinting.

Resumo

A sinalização por FGF está envolvida no controle de vários processos, incluindo proliferação e diferenciação celular, do desenvolvimento embrionário até a homeostase do organismo adulto. Mutações de ganho de função em FGFR2 são causadores de várias doenças congênitas do esqueleto, incluindo a Síndrome de Apert (SA). Usando células-tronco mesenquimais (MSC) que abrigam a mutação para SA, S252W, obtidos a partir do periósteo, nós avaliamos o papel de sinalização FGF na proliferação, migração e diferenciação osteogênica e os comparamos aos fibroblastos obtidos a partir do mesmo tecido desses indivíduos. As curvas de crescimento apresentaram efeitos opostos da mesma mutação nesses tipos de células diferentes: enquanto MSCs S252W proliferam menos, fibroblastos S252W crescem muito mais rápido. Em meio pobre de SFB, a maior migração foi observada em fibroblastos S252W mas não em MSCs S252W. FGFR2^{S252W} induz aumento da diferenciação osteogênica em ambos os tipos celulares estudados, mas a diferença em comparação com as células selvagens foi mais acentuada em fibroblastos SA, que foi revertida pela inibição de JNK. Também demonstramos que a sinalização pelos fibroblastos S252W aumenta a diferenciação osteogênica de MSCs do periósteo, mas surpreendentemente não de MSCs de outros tecidos. Em conclusão, as MSCs e fibroblastos responderam diferentemente aos efeitos patogênicos da mutação. Enquanto o excesso de sinalização FGF devido à FGFR2^{S252W} teve efeito mais drástico sobre os fibroblastos, também tornou os fibroblastos mais influentes sobre as MSCs do que o contrário. Além disso, os fibroblastos SA sinalizam somente para células MSCs da mesma origem, salientando que as MSCs de nichos diferentes poderiam apresentar um imprinting de genes específicos.

Introduction

The mammalian Fibroblast Growth Factors (FGFs) are a family of 18 extracellular ligands characterized by a conserved core of 120 amino acids and their strong affinity for heparin sulphate (Ornitz, 2000). They have been identified in several multicellular organisms, from *Caenorhabditis elegans* to *Homo sapiens* (Itoh & Ornitz, 2004). Since the discovery of the FGFs in 1973 (Armelin, 1973), these molecules and their four receptors (FGFRs) have been linked to a variety of biological processes, from early development to homeostasis of the adult organism, with a wide variety of mitogenic, regulatory, morphological and endocrine effects (Beenken & Mohammadi, 2009). By means of alternate mRNA splicing, epithelial ('b') and mesenchymal ('c') isoforms of FGFR1, FGFR2 and FGFR3 can be expressed. Furthermore, each isoform of the receptor binds to a specific subset of FGFs (Ornitz, 2000). The immediate effect of the phosphorylation of different tyrosine kinase domains of the FGFR2 receptor is well described (Zhang et al., 2009; Bae & Schlessinger, 2010), but the final effects in the cellular and individual phenotype are not well understood although gain-of-function mutations in *FGFR1*, *FGFR2* and *FGFR3* are causative of cancer and several congenital skeletal disorders, such as craniosynostosis (including Apert Syndrome) and dwarfism syndromes.

Apert Syndrome (AS) is an autosomal dominant syndrome mainly caused by the mutations S252W (the most prevalent one, accounting for approximately 60% of the cases) or P253R in the FGFR2 gene. In the presence of these mutations, FGFR2 shows enhanced ligand binding affinity to FGF2 and loses isoform ligand specificity for most of the ligands (Ibrahimi et al., 2001). Since it is located between the second and third immunoglobulin-like domain, it affects both the epithelial and the mesenchymal FGFR2 isoforms. This syndrome is characterized by premature fusion of the coronal sutures, severe syndactyly of the hands and feet and by a range of skeletal abnormalities (Cohen & Kreiborg, 1993d). It is considered the most severe form of craniosynostosis (Cohen, 2000), a condition in which one or more of cranial sutures prematurely fuses by ossification. Although most of the clinical features of AS is a result of signaling disturbance during embryonic development, FGFR2 mutations S252W and P253R also interfere in post natal organism homeostasis. The excessive and repetitive closure of the coronal suture after cranial surgical intervention (Johnson, 2003; Foster et al., 2008), a mandatory procedure for the rehabilitation of these patients, makes this invasive technique needed for more than 10 times from birth until adulthood (Cohen, 2000). The premature suture fusion and the resynostosis process after surgical interventions are not likely the result of alterations in one particular cell type as osteoblasts but the result of perturbations in signaling and in interactions between different cell types and tissues of the cranial suture complex (Slater et al., 2008).

The overlying periosteum of the calvaria, the osteogenic fronts of the bone plates, the intervening mesenchyme and the underlying dura mater forms the cranial suture complex. This complex allows skull deformation during birth, expansion during brain growth and regulates the balance between osteogenic precursors proliferation and differentiation (Slater et al., 2008). Several studies have pointed out dura mater as the major tissue regulating suture patency, which Apert patients would in opposition to contribute to premature embryonic synostosis as well as adult resynostosis (Cohen, 2000; Foster et al., 2008). However, remarkably, many studies have underlined the importance of the periosteum in cranial bone formation, since its removal diminishes calcification of cranial defects in animal models (Hopper et al., 2001; Ozerdem et al., 2003). The periosteum contributes not only to normal bone growth, but also to bone healing, and regeneration (Ito et al., 2001; Orwoll, 2006). It is highly cellular, containing multipotent mesenchymal stem cells, fibroblasts, differentiated osteogenic progenitor cells and osteoblasts (Squier et al., 1990; Allen et al., 2004), and acts as a major source of skeletal stem cells/progenitors during bone repair (Colnot, 2009). Moreover, skeletal abnormalities, such as joint progressive limitation, are consistently reported (Cohen, 2000; McHugh et al., 2007; Murnaghan et al., 2007). It is thus possible that periosteum cells also have a major contribution in the premature suture ossification in Apert patients, and possibly not only in cranial sutures. The precise involvement of periosteum in premature suture ossification is very poorly characterized. In addition, it is still unknown how the different cell types from the calvarial periosteum interact and which is the functional effect of the mutation in these cells.

It has been postulated that the gain of function mutation in *FGFR2*, including the S252W, confers a high proliferative cell capacity (reviewd by Benson & Opperman, 2011) which would account for the higher rate of ossification at the sutures, however, to date it is not known if this new cell property is observed in all cell types. Most of the studies, have been concentrated in murine osteoblasts harboring AS mutation, but both increased (Yang et al., 2008) and decreased (Lomri et al., 1998) proliferation have been observed. Studies on their osteogenic potential has also been conducted and again contradictory results have been observed (Lomri et al., 1998; Mansukhani et al., 2000; Yang et al., 2008) . There are very few studies conducted to evaluate the functional effect of this mutation in human cells, which is very important to be done considering the differences in cell signaling between mice and humans. We have conducted a preliminary study in which we suggested that AS fibroblasts have an increased bone differentiation potential (Fanganiello et al., 2007), which we considered important to be replicated. On the other hand, there are no studies on AS mesenchymal stem cells, the possible precursors of osteoblasts, and if there is interplay between fibroblasts and MSCs of the periosteum.

Therefore, we have conducted this study to confirm if the S252W mutation confers an increased proliferative capacity in fibroblasts and which would be its effect in mesenchymal stem cells,

both cell types from the periosteum overlying the affected suture in Apert syndrome. We also investigated the effect of the S252W FGFR2 mutation in cell motility and cell differentiation of these two cell types and if there is a functional interaction between them.

Results

Characterization of the immunophenotype

We performed flow cytometry experiments with different markers in order to characterize the immunophenotype of fibroblasts and mesenchymal stem cell (MSCs) cultures (Table II.1). Cells with or without S252W mutation were highly positive for mesenchymal cell markers (>90%) and negative for hematopoietic and endothelial cell markers. These results thus show that these cells are of mesenchymal origin.

Table II.1. Percentage of positive cells for mesenchymal (SH3, CD90, CD29), hematopoietic (CD117 and CD45) and endothelial (CD31) cell lines antibodies. The cell filled with an X indicates that cytometry for this antibody was not performed.

	MSCs		Fibroblasts	
	S252W (n=2)	WT (n=2)	S252W (n=4)	WT (n=4)
SH3	96.32-99.78%	94.7-98.68%	78.92-98.45%	93.92-97.87%
CD90	94.92-95.52%	94.02-98.7%	89.12-99.7%	89.46-98.94%
CD29	98.48-99.12%	88.1-98.98%	83.14-99.02%	70-99.25%
CD31	<5%	<5%	<5%	<5%
CD117	<5%	<5%	<5%	<5%
CD45	<5%	<5%	<5%	<5%

Cell proliferation and cell migration

To determine the outcome of FGFR2 promiscuity in periosteal cells, we first explored the changes in cell proliferation in WT and S252W FGFR2 in fibroblasts and MSCs from this tissue. S252W mutation increased cell proliferation in fibroblasts at all times of culture (24h: $p = 0.0002$, 48 h: $p = 0.0002$; 72h: $p = 0.0002$) (Figure II.1A) and in different culture conditions (0.5% FBS medium: $p=0.014$; 10% FBS medium: $p=0.04$; and 20% FBS medium: $p=0.0001$) (Figure II.1C). On the other hand, in MSCs, the mutation decreased cell proliferation after 72h of culture (72 h: $p = 0.0018$) (Figure II.1B) and in enriched medium (20% FBS medium: $p=0.0041$) (Figure II.1D).

We next evaluated the effect of FGFR2^{S252W} in cell migration. Mutant receptor increased cell migration in fibroblasts only in restrictive medium condition (0.5% FBS medium: $p<0.0001$) (Figure II.1E), but had no effect in MSCs (Figure II.1F).

Figure II.1. (A) Comparative analysis of the proliferation of WT (from 3 individuals) and S252W (from 3 patients) fibroblasts and (B) WT (from 3 individuals) and S252W (from 3 patients) MSCs. Each point indicates the average for each time and each condition and the error bars represent the standard deviation for the biological replicate. Analysis of (C) WT (from 3 individuals) and S252W (from 3 patients) fibroblasts and (D) WT (from 3 individuals) and S252W (from 3 patients) MSCs when grown in culture medium with different FBS concentration. Each point indicates the average of each medium after 48h and the error bars represent the standard deviation for the biological replicate. (E) Wound healing assay of FGFR2^{+/S252W} (n = 3) and WT (n = 3) fibroblasts and (F) and FGFR2^{+/S252W} (n = 3) and WT (n = 3) MSCs in high FBS and low FBS growth medium. The bars represent the average number of cells that migrated toward the wound after 12h for each condition and error bars represent the standard deviation for the biological replicate (*: p < 0.05, **: p < 0.01, ***: p < 0.001).

Osteogenic differentiation in vitro and in vivo

Next, we assessed the effects of S252W mutation on osteogenic differentiation of these cells. We performed experiments on key points of *in vitro* differentiation protocol in order to evaluate if the mutation has more influence on a specific period of osteoblastic differentiation.

The ninth day of differentiation is ideal to access the levels of alkaline phosphatase (ALP) in cultures, as in this period occurs the peak production of the enzyme. ALP provides the phosphate needed for the production of matrix calcium. S252W fibroblasts showed 6-fold increase in ALP activity in comparison to WT fibroblasts ($p < 0.0001$) (Figure II.2A), while S252W MSCs had 3-fold increase in comparison to WT MSCs ($p = 0.0002$) (Figure II.2B).

After two weeks in differentiation medium, we analyzed initial calcium deposition in the extracellular matrix (ECM) through Alizarin red staining. S252W fibroblasts showed 2.7-fold increase in ECM calcium in comparison WT fibroblasts ($p < 0.0001$) (Figure II.2C), while S252W MSCs had 1.5-fold increase in comparison to WT MSCs ($p = 0.016$) (Figure II.2D).

Finally, we evaluated concentration of ECM calcium at the end of differentiation (21st day). S252W fibroblasts showed 1.7-fold increase in ECM calcium in comparison to WT fibroblasts ($p = 0.002$) (Figure II.2E), while S252W MSCs had 1.5-fold increase in comparison to WT MSCs ($p = 0.0002$) (Figure II.2F).

In order to validate these data *in vivo*, we performed the bilateral cranial critical defect model using Wistar NIS rats previously described by our group (de Mendonca Costa et al., 2008). At the right side defect we introduced S252W or WT cells associated with biomaterial and at the left side defect we introduced biomaterial only as an internal control of each animal's osteoregeneration. After 4 weeks of the surgery, the right side ossification: left side ossification ratio was 2.6-fold higher when mutant fibroblasts were associated to the biomaterial, instead of WT fibroblasts ($p = 0.03$) (Figure II.2G).

Interactions between periosteal MSCs and fibroblasts during osteogenic differentiation

As shown above, we have observed that the cellular phenotype alterations due to S252W mutation seemed more drastic in fibroblasts than in MSCs, and that S252W fibroblasts are particu-

Figure II.2. S252W and WT fibroblasts and MSCs (all conditions: n = 3) in response to osteogenic medium during different phases of osteogenic differentiation. (A, B) Analysis of alkaline phosphatase activity on the 9th day of osteogenic differentiation. (C, D) Alizarin red S staining quantification at the 14th day of differentiation (E, F) Alizarin red S staining quantification at the 21st day of osteogenic differentiation. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate. (G) Percentage of ossification area of calvarial defects with WT or S252W fibroblasts in rats after 4 weeks of surgery (*: p<0.05; **: p<0.01; ***: p<0.001).

larly more prone to osteogenic differentiation, which is not an unusual function of these cells. These data thus raises the question whether S252W fibroblasts positively influence osteogenic differentiation of other cells.

Therefore, in order to test the hypothesis that cell population with the S252W mutation alters normal signaling in adjacent cells, we used coculture system to simulate the *in vivo* anatomic link between the fibroblasts and MSCs in the periosteum, allowing the paracrine signaling without physical cell interaction. Apert fibroblasts induced 30% more differentiation of periosteal MSCs, whether WT (n=3) or S252W (n=2), in ALP assay (WT MSCs: p=0.0003; S252W MSCs: p=0.04) and alizarin red staining (vs. WT MSCs: p=0.007) (Fig. II.3A ad II.3C). Interestingly, AS fibroblasts did not induce bone ossification of mesenchymal stem cell from other tissue, such as dental pulp stem cells (DPSC) (Figure II.3A ad II.3C). Further, S252W MSCs and WT MSCs exhibit no influence on the osteogenic differentiation of S252W fibroblasts (Figure II.3B and II.3D).

Figure II.3. Effects of interaction between periosteal MSCs and fibroblasts. (A) Analysis of alkaline phosphatase activity on the 9th day and (C) Alizarin red S staining at the 21st day of osteogenic differentiation of Apert MSCs (n = 2), WT MSCs (n = 2), WT DPSC (n = 1) and S252W fibroblast (n = 1) co-cultured with fibroblasts with or without the mutation in the presence of osteogenic medium. (B) Analysis of alkaline phosphatase activity on the 9th day and (D) Alizarin red S staining at the 21st day of osteogenic differentiation of Apert fibroblast (n = 1) co-cultured with MSCs with or without the mutation in the presence of osteogenic medium. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate (*: p < 0.05, **: p < 0.01, ***: p < 0.001).

Potential molecule involved in altered fibroblast osteopotential

JNK (c-Jun N-terminal kinases) has been reported as crucial for the final stage of differentiation in pre-osteoblasts and pluripotent cells (Gallea et al., 2001; Celil & Campbell, 2005; Matsuguchi et al., 2009; Liu et al., 2010), and is considered a critical regulator of osteocalcin and osteopontin (Guicheux et al., 2003; Matsuguchi et al., 2009). In addition, two members of MKPs subgroup (mitogen-activated protein kinase phosphatases), negative regulator of JNK activity (Patterson et al., 2009), are associated to craniosynostosis: DUSP6 (Li et al., 2007) and DUSP2 (Fanganiello et al., 2007). The loss of *Dusp6* leads coronal craniosynostosis in mice and we previously reported that *DUSP2* was one of the most significant differentially expressed genes in Apert syndrome periosteal cells. Therefore, JNK is an interesting candidate for altered osteogenic potential in S252W fibroblasts.

We treated S252W fibroblasts with 2 μ M (IC₅₀) (Joiakim et al., 2003) and 4 μ M (twice IC₅₀) of SP600125, JNK phosphorylation inhibitor during osteogenic differentiation protocol and observed a lower ALP activity as we increased the concentration of SP600125 (untreated vs. +2 μ M SP600125: $p=0.025$; vs. +2 μ M SP600125 vs. +4 μ M SP600125: $p=0.014$) (Figure II.4). At the maximal inhibition of JNK, ALP activity of S252W fibroblast and WT fibroblasts were equivalent. Hence inhibition of JNK activity rescued the altered osteogenic potential of Apert fibroblasts.

Figure II.4. Effects of JNK inhibition in periosteal S252W (n=2) and WT (n=2) fibroblasts. Analysis of alkaline phosphatase activity on the 9th day of osteogenic differentiation. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Discussion

The only treatment available nowadays for Apert Syndrome patients is surgical intervention which consists of artificial reconstruction of the coronal sutures (Posnick et al., 1995). Nevertheless, post-surgical ossification of the sutures is frequent and any surgical treatment for craniosynostosis during childhood is considered a procedure to delay but not to prevent synostosis (Cohen, 2000). To understand and provide a way to prevent resynostosis of the sutures so that they remain opened during the growth phase of the individual would enhance life quality of these patients. Bone regeneration studies have enrolled the periosteum as a significant contributor to this process (Ito et al., 2001; Orwoll, 2006), not only through molecular signaling but mainly by providing osteoprogenitor cells (Colnot, 2009), but, their contribution to the premature suture ossification in Apert or other craniosynostosis due to gain of function mutations in FGFR2 is not clear. Unbalanced FGF signaling can interfere in osteoprogenitor cells from the periosteum and contribute to resynostosis, though this process continues to be poorly understood. In order to verify if the mutation S252W FGFR2 confers the same properties in fibroblast and mesenchymal stem cells of the periosteum, we have established cell cultures of these two cell types from affected and control individuals. We were not able to distinguish these two cell types based on positive staining for mesenchymal cell markers or cell morphology, as expected (De Bari et al., 2006). However, the positive bone differentiation seen in WT MSCs but not in WT fibroblasts confirm that our protocols allow the establishment of two distinct cell populations, as we have shown in our previous reports (Fanganiello et al., 2007; Bueno et al., 2009).

The S252W mutation positively influenced cell proliferation in fibroblasts, even in critical culture conditions, such as 0.5% FBS medium. Yet it had a negative effect on MSCs, but only in ideal culture environment. Literature data in regard to the presence of FGFR2^{S252W} and cell proliferation are controversial. Some studies that investigated murine osteoblasts (Yang et al., 2008), murine stem cells (Miraoui et al., 2009), human fibroblasts of periosteum (Fanganiello et al., 2007) or calvarial cells during embryonic formation of sutures in Apert mouse models (Wang et al., 2005; Holmes et al., 2009) point S252W mutation as responsible for increasing cell proliferation. However, studies with human calvarial pre-osteoblasts (Lomri et al., 1998) and osteoblasts obtained from one of the Apert mouse model (Chen et al., 2003) showed that S252W mutant cells showed no differences in cell growth compared to wild type cells. Interestingly, the murine MSCs with FGFR3 mutation proliferate less than the wild type cells (Su et al., 2010). Although we cannot rule out differences in used protocols, our results suggest that the effect of the mutation on cell proliferation is dependent on the cell type under analysis, which would explain the controversy in the literature.

Through the wound healing assay, we show that, FGFR2^{S252W} had effect on the migratory property of fibroblasts, but not on MSCs. This effect on the fibroblasts, however, were dependent on FBS availability, FBS acts as a culture medium supplements that provides not only growth factors, but also cellular growth inhibitors (Freshney, 2005). It was previously reported that FGFR2^{S252W} has enhanced affinity by different FGFs (Ibrahimi et al., 2004), therefore, the altered AS cell proliferation and cell migration in response to different FBS concentration indicates that FBS contains growth stimulating FGFs to which S252W fibroblasts are more sensitive to and FGFs that in high concentration inhibit migration of S252W fibroblasts.

Regarding the osteogenic potential, we found that FGFR2^{S252W} induces increased osteogenic differentiation in both studied cell types, especially at the early stages. Although studies investigating osteoblasts obtained from Apert mouse (Chen et al., 2003) and murine osteoblasts transfected with mutated receptor (Mansukhani et al., 2000) indicated the osteogenic potential as unchanged or even inhibited by the mutation, most literature results, ranging from studies with murine stem cells to human pre-osteoblasts transfected with FGFR2^{S252W}, are consistent with our results (Lomri et al., 1998; Lemonnier et al., 2001a; Yang et al., 2008; Holmes et al., 2009; Miraoui et al., 2009).

It has been established the need of a dynamic system based on integrated signals between stem cells and cells from their surrounding niche, such as fibroblasts, to maintain tissue physiology (Scadden, 2006). Thus, it was very important to evaluate the effect of the mutated FGFR2 fibroblast over the stem mesenchymal cells, the expected osteoblast precursors. Our coculture assay, interestingly, showed that the presence of Apert fibroblasts positively influences osteogenic differentiation of MSCs or osteoblasts, with or without the mutation, given that the latter are originated from the same tissue as the fibroblasts. These results taken together with the increased proliferation and migratory properties of fibroblasts allow us to suggest that periosteum cells might contribute to premature suture fusion in these patients, a property that has mainly been attributed to dura mater (Ang et al., 2010). In addition, our data suggest that the cells we worked with must still harbor regulatory and interactional network program according to the tissue from which they are extracted and that is not erased in cell culture.

The aberrant osteogenic potential in Apert fibroblasts seem to be related to pJNK, since JNK inhibition reverted this phenotype. Shukla et al. demonstrated that ERK inhibition via small molecular inhibitor and RNA interference reverted craniosynostosis in the pathophysiology of the craniosynostosis of the Fgfr2^{+/S252W} mouse (Shukla et al., 2007). It is known that ERK indirectly increases JNK activity (Lopez-Bergami et al., 2007). Our findings suggest that this ERK-JNK pathway

is also perturbed in human patients and that the key molecule involved in craniosynostosis is downstream to ERK and to JNK. Altogether, we propose that identifying this key molecule have a better therapeutic potential in surgical treatment of Apert syndrome patients than ERK and JNK inhibitors, as these kinases are involved in important signaling pathway in the whole body and their inhibition would cause severe side effects.

In summary, our results indicate that fibroblasts extracted from the periosteum of cranial sutures are an important element to promote premature suture closure of AS patients, at least in the post-natal period (Figure II.5). It is also possible that they play an important role in the bone premature ossification during embryo development. Another evidence of the relevance of fibroblasts in Apert syndrome is that, though S252W mutation enhances osteogenic potential of both fibroblasts and MSCs, Apert fibroblasts stimulates osteogenic differentiation in MSCs, but the opposite does not occur (Figure II.5). To better understand the molecular mechanisms underlying our findings, it is crucial to look for molecules secreted by S252W fibroblasts that may contribute to intensify bone differentiation in other cells and whether they are related to the JNK pathway. These molecules could lead to identify candidate drugs that could ameliorate the surgical prognosis of these patients.

Figure II.5. In the periosteum (pink) overlying the suture (brown), fibroblasts (green cells) and MSCs (blue cells) have similar cell growth and cell migration rate. However, fibroblasts exhibit low osteogenic potential while MSCs show higher osteopotential (left). The S252W mutation has a positive effect on both proliferation and migration of fibroblast, and a negative effect on MSCs proliferation. It has no consequence on MSCs migration. Both cell types have enhanced osteogenic differentiation and S252W fibroblasts show positive influence on MSCs differentiation. Inhibition of JNK phosphorylation by SP600125 can null the effect of the mutation over osteodifferentiation of fibroblasts.

Materials and Methods

Subjects

Coronal suture periosteal fibroblasts from three AS patients (FGFR2^{+/S252W}) and from three age- and sex-matched control subjects were obtained as described earlier by us (Fanganiello et al., 2007). The presence of the p.S252W mutation was confirmed by direct DNA sequencing and expression of FGFR2c in the primary fibroblasts was examined by Western Blot and RT-PCR (Fanganiello et al., 2007).

Cell Culture

Periosteum harvested from the AS patients or control individuals were split in half for fibroblast and mesenchymal stem cell extraction. Primary periosteal fibroblasts cells derived from periosteal flaps were grown in fibroblast growth medium (DMEM High-Glucose, 20% fetal bovine serum [Invitrogen] and 2 mmol/L l-glutamine, penicillin, and streptomycin). Cells were passaged at near confluency with trypsin-EDTA. MSC cultures were obtained from finely minced periosteum after 30 minutes trypsin incubation and grown in MSC growth medium (DMEM-F12 [Invitrogen] supplemented with 15% Fetal Bovine Serum (FBS, HyClone) and 2 mmol/L l-glutamine, penicillin, and streptomycin). All cells were cultured in a humidified incubator at 37°C and 5% CO₂. All tests were performed between the third and the fifth subculture.

For each cell line we performed experiments in technical triplicates. For all the experiments we performed, we used all three cell line for each condition, when not it is indicated by “n” value. Thus we tried to ensure that the results we obtained were representative of the biological variance we see in human patients.

Immunophenotyping

To analyze cell-surface expression of typical protein markers, adherent cells were incubated with the following anti-human primary antibodies: CD29-PECy5, CD31-phycoerythrin (PE), CD45-fluorescein isothiocyanate (FITC), CD90-R-PE, CD117-PE, and SH3 (Becton Dickinson). A total of 5,000 labeled cells were analyzed using a Guava EasyCyte flow Cytometer running Guava ExpressPlus software (Guava Technologies).

Cell proliferation analysis

A concentration of 4,000 cells/cm² was plated to each well of a 12-well flat bottom plate in fibroblast growth medium. After 24h, when total cell adhesion was verified, the fibroblasts were starved for another 24h and MSCs for 48h. At the initial time point (0h), we changed the starvation medium for the respective cell growth medium or starvation medium supplemented with 0.5%, 10% and 20% FBS. At the indicated times, the cells were trypsinized and counted using Guava EasyCyte Flow Cytometer (Guava Technologies). The experiment was done in triplicates for each time point and each cell line.

In vitro Wound healing assay

We plated the cells (3x10⁵) on 12-well culture plates (Corning) in growth medium. When cells reached 100% confluence, the fibroblasts were starved for another 24h and MSCs for 48h. After starvation, a single wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The cell layer was then scratched with a P-200 pipette tip, the debris was removed by washing with PBS and we added growth medium. Photographs of the wound adjacent to reference lines scraped on the bottom of the plate were taken using an Axio Observer microscope under 5× field (Zeiss) at 0h and 12h after the injury as done. We used the ImageJ software (Rasband, 1997-2009) and Adobe Photoshop CS3 (Adobe) to analyze and calculate the number of cells that moved into the wound. The experiment was done in triplicates for each treatment and each cell line.

Osteogenic differentiation in vitro

To induce osteogenic differentiation, periosteal fibroblasts and MSCs from three AS patients and from three controls were plated in 24-well plates (5x10³ cells/cm²) and cultured for three weeks in DMEM High-Glucose, 0.5% FBS, 0.1 mM dexamethasone (Sigma-Aldrich), 50 mM ascorbate-2-phosphate (Sigma-Aldrich Corp., St. Louis, MO), 10 mM β-glycerophosphate (Sigma-Aldrich), 1 percent antibiotic. For the coculture assay, the cells were plated at the same concentration onto 12-mm transwell inserts of 12-well plates, 0.4μm pore size (Corning Costar). Media changes occurred every three to four days. As an internal control of the experiment, the same cells were maintained throughout the 21 days of differentiation in control medium, which consisted of growth medium.

Alkaline phosphatase activity was assessed at the 9th day of differentiation through a biochemical assay. The cells were provided with phosphatase substrate (Sigma-Aldrich) and the resulting p-nitrophenol was measured colorimetrically by the use of a Multiskan EX ELISA plate reader (Thermo Scientific) at 405nm.

After 14 and 21 days, calcified matrix production was analyzed by Alizarin Red S staining and quantification was done as previously described (Gregory et al., 2004).

Osteogenic differentiation in vivo

A 4.5 mm in diameter ceramic scaffold (60% hydroxyapatite and 40% of β -tricalcium phosphate: Cellco Scaffold TM) is moistened with standard medium and mixed with 106 human Apert fibroblasts or mscs. The cells, attached to the biomaterial, are pre-differentiated in osteogenic medium and incubated at 37° C in 5% CO₂ for five days.

The *in vivo* differentiation model used NIS Wistar rat (male, aged 2 months old, weighing a maximum of 200g) and has been described previously by our group (de Mendonca Costa et al., 2008). We used a trephine bur of 4.5 mm in diameter to obtain two cranial critical defects which are made in the parietal region, lateral to the sagittal suture, where two scaffolds were implanted per animal, one side being biomaterial alone (left defect) and the other, biomaterial associated to cells (right defect). The animal was kept in ventilated racks with standard conditions of temperature, ventilation and lighting (22° C, 12 hours light cycling per day) with free access to food and water. After 4 weeks of surgery, the rats are sacrificed in a CO₂ chamber. The calvaria was removed and fixed in 10% formalin for 24 hours and then decalcified in 5% formic acid for 48 hours and embedded in paraffin. Slices were obtained from 5µm and stained with hematoxylin and eosin.

We analyzed three transversal slices of the calvaria of each animal. Ossification area of each defect was calculated through Axio Vision Carl Zeiss based on 10x amplified images obtained from Axio Observer.A1 Carl Zeiss microscope. Percentage of the defect area that ossified at the right side was normalized by the percentage of the defect area that ossified at the left side, so that for each animal we obtained 3 ratio values.

JNK inhibitor treatment

SP600125 (Sigma-Aldrich), an antrapirazolone with molecular weight of 220, is a reversible ATP-competitive JNK inhibitor. Stock solutions of at least 20mM were made using 100% dimethyl sulfoxide. Media were supplemented with 2 μ M and 4 μ M of the inhibitor, which corresponds to IC50 and twice the IC50 respectively (Joiakim et al., 2003).

Statistical analysis

Statistical analysis was performed using the GraphPad InStat software (GraphPad). Continuous variables were expressed by mean and standard deviation and the groups were compared by Student's t-test. A p value < 0.05 was considered statistically significant.

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III. FGF19 Interferes with osteogenic differentiation potential of human periosteal fibroblasts with gain-of-function FGFR2 mutation

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Abstract

Apert syndrome (AS) is characterized by craniosynostosis and limb abnormalities and most of the cases are primarily caused by the S252W mutation in FGFR2, which lead to prolonged ligand-receptor engagement and violation of FGFR2 ligand binding specificity. This mutation compromises not only the embryo development, but also the child after birth, particularly by an intriguingly high rate of post-surgical cranial resynostosis. Despite of a large literature on FGF-FGFR signaling, we still have unanswered questions concerning the effect of this mutation on cellular function and how the mutation ultimately cause this phenotype. By evaluation of cellular behavior in periosteal fibroblasts and MSCs from the coronal suture of AS S252W patients and from matched controls we observed that distinct FGFs have different roles in altered mutant cell behaviors. In the S252W fibroblast, FGF2, -10 and -19 increased the proliferation, FGF2 increased migration and FGF19 slowed osteogenic differentiation. In S252W MSCs, FGF2 increased proliferation, FGF10 increased migration and FGF19 increased the osteogenic differentiation. The osteogenic effect of FGF19 was previously unknown and now builds up the involvement of endocrine FGFs in syndromic craniosynostosis. Overall, the new correlations between FGF2, -10 and -19 and abnormal cellular functions in the presence of the S252W mutation here described is a significant contribution in the understanding of Apert syndrome molecular signaling.

Resumo

A Síndrome de Apert (SA) é caracterizada por craniossinostose e anormalidades de membros e a maior parte dos casos são causados principalmente pela mutação S252W em FGFR2, que levam à ligação FGF-receptor prolongada e violação da especificidade de ligante. Esta mutação não só compromete o desenvolvimento do embrião, mas também na criança após o nascimento, principalmente por uma taxa elevada de ressinostose pós-cirúrgica. Apesar de uma vasta literatura sobre a sinalização FGF-FGFR, ainda há dúvidas a respeito do efeito desta mutação na função celular e como a mutação, em última análise, causa esse fenótipo. Pela avaliação do comportamento celular em fibroblastos e MSCs do periósteo da sutura coronal dos pacientes como S252W e de controles pareados, observamos que FGFs distintos têm papéis diferentes na alteração de comportamento da célula mutante. Nos fibroblastos S252W, FGF2, -10 e -19 estimularam a proliferação, FGF2 aumentou a migração e FGF19 desacelerou a diferenciação osteogênica. Em MSCs S252W, FGF2 induziu maior proliferação, FGF10 aumentou migração e FGF19 acelerou a diferenciação osteogênica. O efeito osteogênico de FGF19 era até então desconhecida e agora aponta a participação de FGFs endócrina em craniossinostoses síndrômicas. Em geral, as novas correlações entre os FGF2, -10 e -19 e funções celulares anormais na presença da mutação S252W aqui descritas formam uma contribuição significativa para a compreensão da sinalização molecular da síndrome de Apert.

Introduction

Apert Syndrome (AS) is a congenital rare disorder that compromises mainly craniofacial and limb bones. It is characterized by premature fusion of the coronal sutures, severe syndactyly of the hands and feet and by a range of skeletal abnormalities (Cohen & Kreiborg, 1993a). Mental deficiency, central nervous system (CNS) alterations and a variety of visceral malformations may also be present (Cohen, 2000; Ladher et al., 2000). AS is an autosomal dominant condition mainly caused by the mutations S252W (the most prevalent one, accounting for approximately 64% of the cases) or P253R in the *FGFR2* gene. Although most of the clinical features are consequences of signaling perturbations during embryonic development, the mutation also interferes in post-natal homeostasis, particularly by excessive ossification and repeated suture closure after cranial surgical intervention, a necessary procedure for the rehabilitation of these patients (Johnson, 2003; Foster et al., 2008).

The signaling of fibroblast growth factors (FGFs) through FGF receptors is pivotal in a plethora of functions, with mitogenic, regulatory, morphological, and endocrine effects. Each of the 18 mammalian FGF is expressed in specific periods and tissues during development. With the exception to FGFs 3-6, all the other FGFs are expressed in mouse cranial sutures in E17.5 (Hajihosseini & Heath, 2002). Having in mind that FGFs are key players not only in development, morphogenesis, angiogenesis, hematopoiesis, and survival, but also in post-natal metabolism, one would expect that different FGFs triggers distinct cell response.

The periosteum signaling is essential for the correct shaping and development of the bones (Ito et al., 2001, Orwoll, 2006), including the calvaria (Hopper et al., 2001; Ozerdem et al., 2003), through coordinated proliferation, cell migration and osteogenic differentiation and as a cell source (Colnot, 2009). In an earlier study (Fanganiello et al., 2007), we described that AS coronal suture fibroblasts presented an increased osteogenic potential as compared to control cells. Later, studying calvarial periosteum cells, we observed that MSCs and fibroblasts responded differently to the pathogenic effects of *FGFR2*^{S252W}, which seemed to have more drastic effect on fibroblasts rather than MSCs. Moreover we proposed that the opposite effects of high and low fetal bovine serum (FBS) medium on cell migration and cell proliferation of S252W cells could be a result of the different influence of distinct FGFs on the mutant receptor.

Thus, the aim of the present study is to disentangle the effects of different FGFs on the cell phenotype in the presence of S252W mutation, through two approaches (1) we performed a screening for FGFs with more striking effect over coronal suture periosteum AS fibroblasts, and (2) analyzed cell proliferation, cell motility and osteogenic differentiation potential of periosteal mutant fibroblasts compared to MSCs.

Results

FGF screening

We chose FGFs that represented most FGFs subfamilies and with increased affinity to FGFR2^{S252W}: FGF2, FGF7, FGF8, FGF9, FGF10, FGF18 and FGF19 (Ibrahimi et al., 2004). Forty-eight hours after plating the same number of cells, only FGF2 treated control fibroblasts showed a significant difference (increase of 25%, $p = 0.023$) compared to untreated ones.

In turn, S252W fibroblast significantly increased cell number when treated with FGF2 (100% increase, $p = 0.014$), FGF10 (75% increase, $p = 0.046$) and FGF19 (125% increase, $p = 0.0042$) (Figure III.1), but not with other FGFs.

Figure III.1. Analysis of fibroblast proliferation control ($n = 1$) (A) and Apert ($n = 1$) (B) when grown in culture medium with different FGFs. Each point indicates the average of each medium after 48h and the error bars represent the standard deviation. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

Cell proliferation

A positive effect on mutant cell proliferation by FGF2, FGF10 and FGF19 was confirmed by performing this assay in three other S252W fibroblast and three WT fibroblasts, all in passage 4 to 5. All mutants showed a significant increase in proliferation in the presence of FGF2, FGF10 or FGF19 (Figure III.2D, E and F), while the only significant increase observed in WT fibroblasts was in presence of FGF2 (Figure III.2A, B and C). The results of the experimental proliferation curve were confirmed by BrdU incorporation assay (Figure III.2L).

Likewise in WT fibroblasts, WT MSCs proliferation was only stimulated when adding FGF2 to the medium (Figure III.2G, H and I). S252W MSCs proliferation was stimulated by both FGF2 and FGF10 (Figure III.2J and K).

Figure III.2. Comparative analysis of cell proliferation assay in response to EGF2, EGF10 and EGF19 in WT fibroblasts (A, B, C), S252 fibroblasts (D, E, F), WT MSCs (G, H, I) and S252W MSCs (J, K) for 72 hours and percentage of BrdU incorporation in these cells after 48 hours. Each point indicates the average for each time point and error bars represent standard deviation of the technical triplicate.

Cell migration

In order to evaluate the role of FGF2, FGF10 and FGF19 on cell migration, we performed an *in vitro* wound healing assay. In WT fibroblasts, no FGF significantly increased the number of cells that migrated (Figure III.3A). Treatment with FGF2 was able to enhance the migration of S252W fibroblasts ($p < 0.0001$), but the addition of FGF10 or FGF19 to the medium had no significant effect (Figure III.3B).

WT MSCs have not had migration rate changed in any of various conditions (Figure III.3C). In S252W MSCs instead, we observed increase in cell migration when exposed to FGF10 ($p < 0.0001$) but there was no differential response to FGF2 nor to FGF19 (Figure III.3D).

Figure III.3. Wound healing assay for WT fibroblasts (n=3) (A), S252W fibroblasts (n=3) (B), WT MSCs (n=3) (C) and S252W MSCs (n=3) (D) in the presence of FGF2, FGF10 and FGF19. The bars represent the average number of cells that migrated toward the wound after 12h for each condition and error bars represent the standard deviation for the biological replicate (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Osteogenic potential in vitro

To examine whether different FGFs may alter the osteogenic potential of control and Apert periosteal fibroblasts, we treated these cells with osteogenic induction medium supplemented with FGF2, FGF10 and FGF19 and performed experiments on key points of *in vitro* differentiation protocol. In WT fibroblasts, we observed no difference among treatments at any step of the osteogenic induction protocol (Figure III.4A, C and E).

At the 9th day of differentiation we accessed alkaline phosphatase (ALP) activity in the cultures. In S252W fibroblasts, the presence of FGFs combined with osteogenic induction medium did not contribute to increased alkaline phosphatase activity (Figure III.4A). Similarly, in WT MSCs, no FGF contributed to alter alkaline phosphatase (Figure III.4B). In S252W MSCs, the presence of FGF2 in the osteoinductive medium increased the enzyme activity by 1.4-fold ($p=0.04$) (Figure III.4B).

We observed that in the second week of osteogenic differentiation, the S252W mutation in fibroblasts led to a reduced production of extracellular matrix calcium by 40% when exposed to FGF2 ($p = 0.004$), FGF10 ($p = 0.01$) and FGF19 ($p = 0.0001$) (Figure III.4C). There was no difference between cultures with different FGFs in the WT MSCs (Figure III.4D). S252W MSCs had higher ECM calcium deposition in 1.5-fold when exposed to FGF19 ($p=0.003$), but not to other FGFs (Figure III.4D).

At the end of the differentiation FGF2 and FGF10 led to a 50% decreased matrix calcium production in S252W fibroblasts (FGF2: $p = 0.03$; FGF10: $p = 0.005$) as compared to untreated cells, but there was a similar amount of calcified matrix in the presence of FGF19 when compared to untreated AS cells (Figure III.4E). In WT MSCs, alizarin red staining remained the same in all samples (Figure III.4F). In turn, in S252W MSCs, only FGF2 treatment affected calcium deposition with a 40% reduction ($p=0.007$) (Figure III.4C).

We also analyzed the gene expression of osteogenesis markers. All markers showed basal expression in WT fibroblasts during differentiation (Figure III.5). RUNX2 is a transcription factor essential for the initial period of differentiation of osteoblasts from progenitor cells (Franceschi and Xiao, 2003), it positively regulates the expression of several osteoblast specific genes such as collagen type 1 and osteocalcin (Komori et al., 1997; Ducy et al., 1999). In mutant fibroblasts without FGF, or with FGF2 or FGF19, *RUNX2* expression reached its peak at the second week of differentiation, which is not observed when treated with FGF10 (Figure III.5A). In WT MSCs, the maximum expression of all conditions was observed towards the end of differentiation protocol and the highest peak

Figure IV.4. S252W and WT fibroblasts and MSCs (all conditions: n = 3) in response to osteogenic medium supplemented with FGF2, FGF10 or FGF19 during different time points of osteogenic differentiation. (A, B) Analysis of alkaline phosphatase activity on the 9th day of osteogenic differentiation. (C, D) Alizarin red S staining quantification at the 14th day of differentiation (E, F) Alizarin red S staining quantification at the 21st day of osteogenic differentiation. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate (*: p<0.05; **: p<0.01; ***: p<0.001).

occurred when cells were treated with FGF2 (Figure III.6A). RUNX2 expression profile in S252W MSCs was similar to the one seen in WT MSCs, yet, its expression was always higher in mutant cells. FGF2 treated mutant MSCs were an exception, since the expression hits the highest point at week 2 and continues with elevated expression throughout the differentiation process (Figure III.6A).

Type 1 collagen (COL1A1) is a protein that strengthens and supports various tissues, including cartilage, bones, and tendons. Ninety percent of the bone matrix is composed of type 1 collagen. In osteogenic medium without FGF, S252W fibroblasts exhibit a gradual increase in the expression of *COL1A1*, with the maximum at week 3. Adding FGF2 or FGF10 to these cells leads to the uppermost expression at the 7th day of differentiation. Treated with FGF19, this peak shifted to day 14 (Figure III.5B). *COL1A1* has high expression in WT MSCs when undifferentiated, but mRNA levels drops drastically after differentiation induction in untreated and FGF10 treated cells. However, when treated with FGF2 or FGF19, the expression reaches its peak at week 3 (Figure III.6B). In undifferentiated Apert MSCs, *COL1A1* expression is 3.5 times lower than when under osteoinduction. They showed a top expression at the end of the protocol, except in FGF19 treatment, when the peak starts at week 2 and continues until week 3 (Figure III.6B).

The *ALPL* gene encodes alkaline phosphatase, a glycosylated membrane linked enzyme involved in matrix mineralization. It was observed the highest level of expression at the first week in S252W fibroblasts cultured in induction medium without FGF or with FGF2, FGF10, or FGF19 (Figure III.5C), consistent with the previous results on enzyme activity. In WT MSCs, its expression gradually increased during osteogenic differentiation, and the mRNA level was elevated in WT MSCs treated with FGF2 or FGF19 (Figure III.6C). S252W MSCs had a very similar *ALPL* expression profile to that of WT MSCs, but generally higher. In FGF10 treatment, S252W MSCs showed an *ALPL* expression peak at week 1 and 3 (Figure III.6C).

In *in vitro* osteogenic differentiation, osteoblasts showed a dramatic increase in expression of noggin (*NOG*), suggesting a role for it as a negative feedback to limit excessive exposure of cells to BMP signaling (Gazzerro et al., 1998). According to this data, we would expect an increase of *NOG* in the last week of the differentiation *in vitro* process. We observed that untreated S252W fibroblasts showed the highest expression of *NOG* at the last two weeks of induction. When treated with FGF2, this peak is limited to the last week, while when treated with FGF10 or FGF19, *NOG* expression is much higher in the second week of the protocol, but remains eminent till the last week (Figure III.5D). *NOG* showed a basal expression in WT MSCs throughout differentiation protocol (Figure III.6D). In turn, S252W MSCs, which presents an enhanced osteogenic potential, had high levels of *NOG* mRNA

when undifferentiated, and this level drops during differentiation, but remains always higher than in WT MSCs (Figure III.6D).

Osteocalcin (BGLAP) is a sialoprotein found in bone, only secreted by osteoblasts. It is produced in the late stages of osteogenic differentiation and is known to inhibit mineralization (Romberg et al., 1986; Hauschka and Wians, 1989). In S252W fibroblasts in osteogenic medium without FGF, we observed a gradual increase in the expression of this marker. In the presence of FGF2 these cells have a similar expression to that of WT fibroblasts with a 4-fold increase in the last week of induction. Treated with FGF10, S252W fibroblasts displayed a high point at week 1, with an abrupt decrease at week 2 and the upregulation of osteocalcin mRNA levels at week 3. At last, addition of FGF19 to mutant fibroblasts during osteogenic differentiation leads to a gradual increase in the expression of this gene throughout the protocol, which becomes constant during the last two weeks (Figure III.5E). In WT MSCs, osteocalcin expression is high before and after the differentiation protocol, meanwhile at week 1 and 2 the expression was minimum (Figure III.6E). In untreated and FGF2 treated S252W MSCs, osteocalcin expression profile was analogous to the one seen in WT MSCs, yet mRNA levels were more elevated, mainly in the presence of FGF2, in which the highest expression at week 3 was 1.5 times higher than in the same cells without FGF. When Apert MSCs were exposed to FGF10, there was no significant decrease in osteocalcin expression in week 1, and at week 2, the expression increased and remained the same till week 3. In response to FGF19, mutant MSCs increased osteocalcin expression at week 1, followed by a decrease at week 2 and elevating again at week 3.

Thus, in WT fibroblasts, differentiation was minimal, with no positive or negative effect of the presence of the FGFs studied. In S252W fibroblasts, however, both FGF2 and FGF10 treatment showed the same ALP activity as untreated cells and the same lower alizarin red staining along the differentiation. In both treatments, the expression of type 1 collagen, noggin and osteocalcin have showed to be disregulated. In spite of this, there is a lower *RUNX2* expression peak in cells treated with FGF10, but not with FGF2, which illustrates the difference in the molecular mechanism responsible for inhibiting osteogenic differentiation of Apert fibroblasts by these two FGFs.

The FGF19, in turn, delayed the osteogenic differentiation of S252W fibroblasts, evidenced by a smaller alizarin red staining at the 14th day followed by matching the same level of staining at the 21st days compared with the same cells untreated and exposed to the inducer. The maximum expression of *RUNX2* at week 2 was half of the one observed in untreated cells, in addition to the highest expression of type 1 collagen at week 2 instead of week 3, may explain the delay in the osteogenic differentiation in the presence of FGF19.

Figure III.5. qRT-PCR results of osteogenic differentiation markers – *RUNX2* (A), *COL1A1* (B), *ALPL* (C), *NOG* (D) and *BGLAP* (E) – during the three weeks of the differentiation of WT and S252W fibroblasts. The columns represent the relative expression and error bars represent the standard deviation.

Figure III.6. qRT-PCR results of osteogenic differentiation markers – *RUNX2* (A), *COL1A1* (B), *ALPL* (C), *NOG* (D) and *BGLAP* (E) – during the three weeks of the differentiation of WT and S252W MSCs. The columns represent the relative expression and error bars represent the standard deviation.

As in WT fibroblasts, WT MSCs showed no positive or negative influence by any on the FGFs studied in osteogenic differentiation. On the other hand, in S252W MSCs, each ligand had a distinct effect. FGF2 increased ALP activity at the 9th day, did not affect calcium deposition at the 14th day, but led to a lower matrix mineralization at the end. The osteogenic markers with altered expression profile in S252W MSCs treated with FGF2 were *RUNX2* and osteocalcin.

Overall, FGF10 did not interfere in the osteogenic differentiation in Apert MSCs. Notwithstanding, FGF19 did not alter ALP activity at the 9th day, but increased the production of calcified matrix in 50% at the 14th day, until it reached the same level of alizarin red staining as of untreated cells at the 21st day. This indicates that in S252W MSCs, FGF19 accelerated differentiation process, which is corroborated by the highest level of osteocalcin mRNA at week1 instead of week 3.

Osteogenic potential in vivo

We showed for the first time that the Apert fibroblasts exhibit a slower osteogenic differentiation in the presence of FGF19, while it accelerates the same process in Apert MSCs. Given the results above we considered important to validate this difference in osteogenic potential of Apert cells in the presence of FGF19 and, thus, we evaluated the effect of FGF19 in the differentiation of these cells *in vivo*. We performed the bilateral cranial critical defect model using Wistar NIS rats previously described and widely used by our group (de Mendonca Costa et al., 2008).

After 4 weeks of the surgery, defect in the right side of the cranium, where we introduced S252W fibroblasts associated with biomaterial and in the presence of FGF19 containing beads, displayed ossification of 9.4% of the defect area, while the defect of the left side of skull, where we inser-

Figure III.7. Histological cross-section of the parietal bone stained with hematoxylin-eosin viewed at 10X magnification. The black arrows indicate the left side (L) and right (R) bone stumps, green arrow points to the sagittal suture. The location of the biomaterials (bluish color) in critical defects is indicated by purple keys. The orange arrows indicate the positions of the ossification isle, whose area was calculated.

ted S252W fibroblasts associated to biomaterial and saline containing beads, ossification represented 3.7% of the defect area ($p=0.02$) (Figure III.7). Therefore, FGF19 treated S252W fibroblasts have lead to a less intense ossification process as compared to the S252W cells without FGF19.

Discussion

To unravel the effects of different FGFs on the periosteal cell phenotype in the presence of FGFR2 mutation, we performed a screening with several FGFs, in which, FGF2, FGF10 and FGF19 induced an increase in S252W fibroblast proliferation and FGF2 and FGF10 induced increase in S252W MSC proliferation. FGF2 and FGF10 results in mutant fibroblasts are in agreement with the results published by Yang et al, 2008 (Yang et al., 2008). On the other hand, only FGF2, which is known to stimulate wild-type FGFR2c receptor (Yu et al., 2000; Ibrahimi et al., 2001; Ibrahimi et al., 2004), had effect on WT fibroblasts and WT MSCs. Interestingly, the proliferation curves of each cell line, whether fibroblast or MSCs, were different but the positive effect of the FGFs was consistent throughout all the cell lines. Compared to the other assays we performed, biological individual variance was more evident in cell proliferation.

This is the first study to show that S252W mutation leads to an increased migration rate of fibroblasts in the presence of FGF2 and of MSCs in the presence of FGF10, which is an important observation since the periosteum may act as a cell source during bone repair (Colnot, 2009). This altered cell property could be related to an excessive signaling of FGFR2c, as increased cell migration has previously been correlated with high expression levels of FGFR2 as well as in the presence of the C342Y mutation, which leads to constitutive activation of FGFR2c (Nomura et al., 2008; Matsunaga et al., 2009; Zhang et al., 2009). It is important to highlight that the same mutation caused fibroblast and MSC to migrate differently in response to different FGFs.

As reported previously by our group (Fanganiello et al., 2007), Apert fibroblasts have enhanced osteogenic potential, which is supported by this study. We are showing here, that the osteogenic induction is however inhibited by the presence of FGF2 in FGFR2^{+/S252W} fibroblast and MSCs or FGF10 in FGFR2^{+/S252W} fibroblast. Therefore, fibroblasts with the S252W mutation seem to share the same functional altered properties as S252W osteoblasts, as FGF2 and FGF10 also inhibit the differentiation process in these cells (Quarto et al., 2008; Yang et al., 2008).

We demonstrate for the first time that Apert fibroblasts exhibit a slower osteogenic differentiation in the presence of FGF19. The *in vivo* results we have achieved are in agreement with the *in vitro* results. In recent years, FGF19 has been more studied for its role as an endocrine FGF in bile acid homeos-

tasis, lipolysis and gall bladder filling (Fu et al., 2004; Tomlinson, 2002; Choi et al., 2006), and, despite FGF23 (from same subfamily of endocrine FGFs) is involved in bone development through phosphate metabolism (Razzaque, 2009; Ohkido et al.), this is the first time that FGF19 is related to osteogenic differentiation. FGF19 is a circulating FGF that can activate FGFR1c, 2c, 3c, and 4 in the presence of Klotho β (Kurosu et al., 2007). This unexpected association of FGF19 in bone formation in Apert cells evidences a possible important role for this FGF in the recurrent post-surgical resynostosis of the sutures.

The change in proliferation, migration and differentiation of cells from the periosteum has important functional implications in setting limits to the suture during embryonic development, an important requirement to ensure proper sutures closure (Merrill et al., 2006). Furthermore, after birth, these altered cellular properties may speed healing and bone formation after surgery, a procedure regularly carried out and indicated in the rehabilitation of AS patients. In summary, our results further highlights the different cell phenotypes carried out by the same mutation in fibroblasts and MSCs extracted from the same tissue that we observed previously. Enrolling distinct FGFs in altered mutant cell behaviors is a further step in the understanding of the altered molecular patterning and signaling in Apert syndrome. The novel osteogenic effect of FGF19 is an important clue to the several abnormalities these patients present throughout the body, since it is circulating FGFs. Future studies on whether FGF19 influence on the mutant cell can be associated to the synostosis promoting ERK-JNK pathway are required.

Materials and methods

Subjects

Coronal suture periosteal fibroblasts from three AS patients (FGFR2^{+/S252W}) and from three age- and sex- matched control subjects were obtained as described earlier by us (Fanganiello et al., 2007). The presence of the S252W mutation was confirmed by direct DNA sequencing and expression of FGFR2c in the primary fibroblasts was examined by Western Blot and RT-PCR (Fanganiello et al., 2007).

Cell culture

Periosteum harvested from the AS patients or control individuals were split in half for fibroblast and mesenchymal stem cell extraction. Primary periosteal fibroblasts cells derived from periosteal flaps were grown in fibroblast growth medium (DMEM High-Glucose, 20% fetal bovine serum (Invitrogen) and 2 mmol/L l-glutamine, penicillin, and streptomycin). Cells were passaged at near

confluency with trypsin-EDTA. MSC cultures were obtained from finely minced periosteum after 30 minutes trypsin incubation and grown in MSC growth medium (DMEM-F12 [Invitrogen] supplemented with 15% Fetal Bovine Serum (FBS, HyClone) and 2 mmol/L l-glutamine, penicillin, and streptomycin). All cells were cultured in a humidified incubator at 37°C and 5% CO₂. All tests were performed between the third and the fifth subculture.

For each cell line we performed experiments in technical triplicates. For all the experiments we performed, we used all three cell line for each condition, when not it is indicated by “n” value. Thus we tried to ensure that the results we obtained were representative of the biological variance we see in human patients.

Exogenous FGF treatment

Periosteal cells from three AS patients and from three control subjects were grown until they reached 80% of confluency. Cells were washed with PBS and then were serum starved for 24h in DMEM not supplemented with FBS. After this period control cells were treated with DMEM High-Glucose, 0.5% FBS and experimental cells were treated with DMEM High-Glucose, 0.5% FBS supplemented with recombinant human FGF2 or FGF10 (PeproTech, Rocky Hill, NJ, USA – diluted in PBS 1X to a final concentration of 2000 pM) or with DMEM High-Glucose, 0.5% FBS supplemented with PBS 1x free of FGFs. When treated with 2000 pM of FGFs (Yu et al., 2000), it was reported similar phosphorylation of both wild type and FGFR2c^{S252W}. Control and experimental cells were harvested at 24h after addition of FGFs, and had its total RNA isolated and purified as described below. Besides the biological triplicate, to account individual variability, the experiments were performed in technical triplicate for each sample, in order to consider possible experimental variability.

Cell-proliferation analysis

A concentration of 4,000 cells/cm² was plated to each well of a 12-well flat bottom plate in fibroblast growth medium. After 24h, when total cell adhesion was verified, the cells were starved for another 24h. At the initial time point (0h), we changed the starvation medium for FGF treatment medium (80% DMEM, 0.5% fetal bovine serum, 2 mmol/L l-glutamine, penicillin, and streptomycin and 36 ng/ml of the respective FGF or no FGF), or for fibroblast growth medium. At the indicated times, the cells were trypsinized and counted using Guava EasyCyte Flow Cytometer (Guava Technologies). The experiment was done in triplicates for each time point and each cell line.

In vitro wound healing assay

Cells (3×10^5) were plated on 12-well culture plates (Corning Incorporated, Acton, MA, USA) in fibroblast growth medium. Cells were starved for 24h when the cell population reached 100% confluence. After starvation, a single wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The cell layer was then scratched with a P-200 pipette tip, the debris was removed by washing with PBS and we added FGF treatment medium (80% DMEM, 0.5% fetal bovine serum, 2 mmol/L l-glutamine, penicillin, and streptomycin and 36 ng/ml of the respective FGF or no FGF) to each well. Photographs of the wound adjacent to reference lines scraped on the bottom of the plate were taken using an Axio Observer microscope under 5× field (Carl Zeiss, Thornwood, NY, USA) at 0h and 12h after the injury. We used the ImageJ software (Rasband, 1997-2009) and Adobe Photoshop CS3 (Adobe) to analyze and calculate the number of cells that moved into the wound. The experiment was done in triplicates for each treatment and each cell line.

Osteogenic differentiation in vitro

To induce osteogenic differentiation, periosteal fibroblasts from three AS patients and from three controls were plated in 24-well plates (5×10^3 cells/cm²) and cultured for three weeks in DMEM High-Glucose, 0.5% FBS, 0.1 mM dexamethasone (Sigma-Aldrich Corp., St. Louis, MO), 50 mM ascorbate-2-phosphate (Sigma-Aldrich Corp., St. Louis, MO), 10 mM β-glycerophosphate (Sigma-Aldrich Corp., St. Louis, MO), 1 percent antibiotic, supplemented with 20 uL of PBS (“untreated”), FGF2 or FGF10 (corresponding to 36 ng of growth factor per mL of medium). Media changes occurred every three to four days. As an internal control of the experiment, the same cells were maintained throughout the 21 days of differentiation in control medium, which consisted of DMEM High-Glucose, 0.5% FBS, 1 percent antibiotic.

Phosphatase alkaline activity was assessed at the 9th day of differentiation through a biochemical assay. The cells were provided with phosphatase substrate (Sigma-Aldrich Corp., St. Louis, MO) and the resulting p-nitrophenol was measured colorimetrically by the use of a Multiskan EX ELISA plate reader (Thermo Scientific, Waltham, Massachusetts, USA) at 405nm.

After 14 and 21 days, calcified matrix production was analyzed by Alizarin Red S staining and quantification as previously described (Gregory et al., 2004).

RNA extraction

Total RNA was isolated from FGF treated and untreated cells at day 7, day 14 and day 21 during osteogenic differentiation using Nucleospin RNA kit (Macherey-Nagel). RNA quality and concentration were accessed respectively by 1.5 percent agarose gel electrophoresis and Nanodrop ND-1000.

Reverse transcription reactions and quantitative real time PCR

Complementary DNA (cDNA) was produced from 2 µg of total RNA using Superscript II reverse transcription kit (Invitrogen). qRT-PCR was performed using approximately 200 ng of cDNA and SYBR Green PCR master mix in an ABI Prism 7500 system (Applied Biosystems). The PCR conditions were: 95°C for 15s, 60°C for 30s, and 72°C for 30s for 40 cycles. Primers were designed with Primer Express software V.2.0 (Applied Biosystems) and the amplification efficiency (E) of each primer was calculated according to the equation $E=10^{(-1/\text{slope})}$. The expression data of the studied transcripts were determined by relative quantification in comparison to four endogenous controls (*GAPDH*, *HMBS*, *HPRT1* and *SDHA*). We verified the gene expression stability of endogenous controls through geNorm VBA applet designed for Microsoft Excel. This tool calculates the most stable reference genes from a set of tested candidate reference genes in a given sample panel, and calculates the gene expression normalization factor for each target sample based on the geometric mean of a user defined number of housekeeping genes (Vandesompele et al., 2002). The expression data is given

Table III.1. Primers used for quantitative real time PCR.

Gene	Foward Primer	Reverse Primer
<i>ALPL</i>	GTGCCAGAGAAAGAGAAAGACCCC	TCTTAGCCACGTTGGTGTGAGC
<i>BGLAP</i>	TTTCAGGAGGCCTATCGGCGCT	ACATCCATAGGGCTGGGAGGTCA
<i>COL1A1</i>	CGAAGACATCCCACCAATCAC	CAGATCACGTCATCGCACAAC
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATG
<i>HMBS</i>	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
<i>HPRT1</i>	TGACACTGGCAAAACAATGC	GTCCTTTTCACCCAGCAAGC
<i>NOG</i>	AGCGCCTAAGCAAGAAGCTGCG	CTTCACGTAGCGCGGCCAAA
<i>RUNX2</i>	ATTACAGACCCCAGGCAGGCAC	AAGACAGCGGGGTGGTAGAGTG

by the ratio between each transcript $\Delta\Delta Ct$ ($E^{\Delta Ct}$) and normalization factor. Primers used in this study are summarized in Table III.1.

To assess the statistical significance of correlation between microarray assay data and qRT-PCR results we used the nonparametric two-tailed Spearman correlation test, with p-values of less than 0.05 considered to be statistically significant.

Osteogenic differentiation in vivo

A 4.5 mm in diameter ceramic scaffold (60% hydroxyapatite and 40% of β -tricalcium phosphate: Cellco Scaffoldex TM) is moistened with standard medium and mixed with 106 human Apert fibroblasts or mscs. The cells, attached to the biomaterial, are pre-differentiated in osteogenic medium with FGF19 (Peprotech, 36 ng/ml) or PBS and incubated at 37° C in 5% CO₂ for five days.

Before surgery, heparin beads (Adar Biotechnologies) are washed in phosphate buffered saline (PBS) and soaked in recombinant human FGF19 (36 ng/ml, Peprotech), or 0.1% albumin bovine serum in PBS for 1 hour at 37°C in agitation (Ladher et al., 2000). These beads are applied directly on the biomaterial immediately before it is inserted into the defect.

The *in vivo* differentiation model used one NIS Wistar rat (male, aged 2 months old, weighing a maximum of 200g) and has been described previously by our group (de Mendonca Costa et al., 2008). We used a trephine bur of 4.5 mm in diameter to obtain two cranial critical defects which are made in the parietal region, lateral to the sagittal suture, where two scaffolds were implanted per animal, one side being biomaterial associated to cells and saline soaked beads (left defect) and the other, biomaterial associated to cells and FGF19-soaked beads (Peprotech) (right defect). The animal was kept in ventilated racks with standard conditions of temperature, ventilation and lighting (22°C, 12 hours light cycling per day) with free access to food and water. After 4 weeks of surgery, the rats are sacrificed in a CO₂ chamber. The calvaria was removed and fixed in 10% formalin for 24 hours and then decalcified in 5% formic acid for 48 hours and embedded in paraffin. Slices were obtained from 5µm and stained with hematoxylin and eosin.

We analyzed three transversal slices of the calvaria of each animal. Ossification area of each defect was calculated through Axio Vision Carl Zeiss based on 10x amplified images obtained from Axio Observer.A1 Carl Zeiss microscope.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat software (GraphPad, California, USA). Continuous variables were expressed by mean and standard deviation and the groups were compared by Student t test. A p value < 0.05 was considered statistically significant.

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IV. Distinct transcriptional circuitry in S252W/FGFR2 human fibroblasts in response to different FGFs

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Abstract

Transmission of extracellular signals via plasma membrane proteins to the intracellular compartment is crucial for the cell to recognize and interrelate with neighboring cells and extracellular structures. Fibroblast growth factor receptors (FGFR) intermediate the signaling from FGFs into the cell. The amplitude of cell response to FGFR signaling is allowed by both alternate mRNA splicing and binding specificity. In the presence of the S252W mutation (Apert syndrome), FGFR2 loses isoform ligand specificity for most of the ligands. We earlier reported a unique expression profile of S252W coronal suture periosteal fibroblasts. In the present study, we aimed to better understand the molecular pathological events taking place in the distinct cell behavior in S252W cells in response to FGF2, FGF10 and FGF19. FGF2, the most abundant FGF in the central nervous system, induced differential expression of genes important for development and maintenance of the CNS only in mutant fibroblasts. Among these genes, we validated *Strc* in Apert mouse brain, evidentiating the role for primary cilia in the establishment of landmark CNS abnormalities of this syndrome. Transcriptome changes induced by FGF10 in S252W cells were significantly associated to immune response. Activation of FGFR2^{S252W} by FGF19 modifies the expression of genes associated to cell proliferation and ossification in conformity with results we previously reported. qRT-PCR analysis of 5 differentially expressed genes in FGF2 treated S252W fibroblasts showed that their expression did not correlate to the one obtained from fibroblasts extracted from Crouzon syndrome patients. In conclusion, the transcriptome circuitry we outlined here is representative of Apert syndrome.

Resumo

A transmissão de sinais extracelulares através de proteínas na membrana plasmática ao compartimento intracelular é fundamental para a célula reconhecer e se relacionar com as células vizinhas e estruturas extracelulares. Receptores de fatores de crescimento de fibroblasto (FGFR) intermediar a sinalização de FGFs na célula. A amplitude da resposta celular à sinalização por FGFR ocorre tanto pelo splicing alternativo do mRNA e pela especificidade de ligação. Na presença da mutação S252W (síndrome de Apert), FGFR2 perde a especificidade ao ligante. Nós anteriormente relatamos um perfil de expressão único de fibroblastos S252W de periósteo da sutura coronal. No presente estudo, buscou-se compreender melhor os eventos patológicos moleculares que ocorrem no comportamento celular em células S252W distintas em resposta a FGF2, FGF10 e FGF19. FGF2, o FGF mais abundante no sistema nervoso central, induziu a expressão diferencial de genes importantes para o desenvolvimento e manutenção do sistema nervoso central só em fibroblastos mutantes. Entre estes genes, validamos *Strc* em cérebro de camundongo Apert, evidenciando o papel de cílios primários no estabelecimento de anomalias no SNC características desta síndrome. Mudanças no transcriptoma induzidas pelo FGF10 em células S252W foram significativamente associados à resposta imune. Ativação de FGFR2^{S252W} por FGF19 modifica a expressão de genes associados à proliferação celular e da ossificação, que está de acordo com os resultados já relatado anteriormente. qRT-PCR, análise de cinco genes diferencialmente expressos em fibroblastos S252W tratados com FGF2 mostrou que sua expressão não se correlacionou com a obtida a partir de fibroblastos extraídos de pacientes com síndrome de Crouzon. Em conclusão, o perfil de expressão que esboçamos aqui é representativo da síndrome de Apert.

Introduction

In order to acknowledge and interact with neighboring cells and extracellular structures, it is essential that the cell conduct extracellular signals via cell membrane proteins to the intracellular compartment. Plasma membrane receptors containing intracellular domains with unique enzymatic, recruiting, or nuclear translocation properties allowed coordinated cell behavior, which is essential to multicellularity. One signal triggers a whole cascade of molecular actions that culminates in modified transcription levels of several genes in the nucleus, which will be responsible for the cellular response to that initial event. Even the smallest perturbation in this arrangement may result in severe consequences for the organism. Fibroblast growth factor receptors (FGFR) intermediate the signaling from fibroblast growth factors (FGFs) into the cell. They are known for their pivotal role in innumerable regulatory and morphological processes since embryonic development to adult homeostasis. The amplitude of cell response to FGFR signaling is allowed by both alternate mRNA splicing, which produces epithelial ('b') and mesenchymal ('c') isoforms of FGFR2; and binding specificity (each receptor binds to a specific subset of FGFs (Ornitz, 2000)).

Mutations that lead to FGFR2 promiscuity are causative of a congenital autosomal dominant rare disorder known as Apert syndrome (AS). Individuals with AS present premature fusion of the coronal sutures, a large defect of the anterior fontanelle, midfacial retrusion, severe syndactyly of upper and lower limbs, a range of skeletal abnormalities, mental deficiency, central nervous system (CNS) alterations and a variety of visceral malformations (Cohen & Kreiborg, 1993d; Cohen, 2000). This syndrome is mainly caused by the mutations S252W (the most prevalent one, accounting for approximately 64% of the cases) or P253R in the *FGFR2* gene.

In the presence of the S252W mutation, FGFR2 shows enhanced ligand binding affinity and loss of isoform ligand specificity for most of the ligands (Ibrahimi et al., 2004). Since it is located between the 2nd and 3rd immunoglobulin domain, it affects both the epithelial and mesenchymal FGFR2 isoforms. Each of the 22 human FGFs is expressed in specific periods and tissues during development. With the exception to FGFs 3-6, all the other FGFs are expressed in mouse cranial sutures in E17.5 (Hajihosseini & Heath, 2002). Therefore in AS patients it has been suggested that enhanced FGFR2 signaling by several FGFs expressed in the cranial suture accounts for the craniosynostosis (Anderson et al., 1998; Wilkie et al., 2002; Ibrahimi et al., 2004) whereas illegitimate binding and signaling of FGFR2c by mesenchymally expressed FGFs is responsible for syndactyly (Ibrahimi et al., 2004). These correlations seem to be very simplistic assumption if we take into account the complexity of the Apert phenotype. It is not yet studied the effect of each FGF in FGFR2 mutated signaling, and its possible consequence to the phenotype.

We previously reported a unique expression profile of S252W coronal suture periosteal fibroblasts from patients as compared to fibroblasts obtained from control individuals (Fanganiello et al., 2007). Also, we reported that FGFR2^{S252W} confers a more drastic gain of function in fibroblasts than in adult mesenchymal stem cells and that FGF2, FGF10 and FGF19 were the ligands that most affected S252W fibroblasts proliferation. Furthermore, these FGFs also modified cell migration and osteogenic differentiation *in vitro* and *in vivo*.

Therefore, we endeavored to better understand the molecular pathological events taking place not only to establish the specific gene expression signature in AS fibroblasts but also in the distinct cell behavior in S252W cells in response to FGF2, FGF10 and FGF19. In order to achieve this goal, we analyzed the transcriptional circuitry triggered by each ligand using gene expression microarray analysis.

Results

Effect of exogenous FGF2 treatment on the transcriptome

Regarding the gene expression profile of wild type cells in response to FGF2, seventy-nine genes were extracted, of which 48 were up regulated and 31 were down regulated (Table IV.1-Anexo). We observed that FGF2 treated WT fibroblasts showed increased expression of genes involved in MAPK signaling pathways (*DUSP6*, *MAP4K4*, *RASA2* and *ITGA2*), PI3K/Akt (*ITGA2*) and Jak-STAT (*IL-13RA2*). The most significant biological features among these DEGS were cell growth (IPA: $p = 0.0002$; DAVID: $p = 0.017$, TG: $p = 0.00024$) and cell motility (IPA: $p = 0.0002$; GT: $p = 0.0005$) (Figure IV.1). Furthermore, IPA analysis found 31 of 79 (39.2%) genes on the same pathway related to movement and cell proliferation network (Figure IV.2A).

We found that upon FGF2 stimulation of mutant fibroblasts significantly increased expression of 21 genes and reduced expression of other genes 34 (Table IV.1-Anexo). Seven (12.7%) of these genes are associated to neurological diseases (*BAT3*, *HS6ST1*, *IFI44L*, *RFC3*, *RPS9*, *STRC* and *TCF19*) according to the IPA software analysis ($p = 0.003$). The most significant biological functions of this set was biosynthetic processes (IPA: $p = 0.01$; GT: $p = 0.03$) (Figure IV.2B). Among these 55 DEGS, 9 (16.4%) were assigned to the same network involved in cell death and cell cycle (Figure IV.2C).

Effect of exogenous FGF10 treatment on the transcriptome

In response to treatment with FGF10, WT fibroblasts showed differential expression of 45 genes -31 up-regulated and 14 downregulated, of which 24 genes were not annotated or non-coding RNAs (Table IV.1 Table IV.1). IPA could build no network with more than 1 DEG. None of the databases used identified a significant cellular function or functional enrichment in control cells treated with FGF10, as expected, since FGF10 does not bind to any of the FGFRs expressed in mesenchymal tissue.

Treatment with FGF10 in S252W cells resulted in 59 DEGS (35 upregulated and 24 down-regulated genes) compared to untreated mutant cells (Table IV.1- Anexo), of which 10 are associated with inflammatory diseases (*HLA-DMA*, *OR12D3*, *MOG*, *RING1*, *TCF19*, *C6ORF15*, *CLIC2*, *LY6G5C*, *POP* and *XCL1*), making immune/inflammatory response the most significant biological function (IPA: $p = 0.0002$; DAVID: $p = 0.03$; GT: $p = 0.0002$) (Figure IV.3A). The gene interaction network built with 8 out of 59 genes was associated with cell cycle and development (Figure IV.B).

Effect of exogenous FGF19 treatment on the transcriptome

A total of 45 differentially expressed genes were found in FGF19 treated WT fibroblasts, of which 29 were upregulated and 16 were downregulated (Table IV.1- Anexo). The most significant cellular function associated with these genes is immune response (IPA: $p = 0.0008$; DAVID: $p = 0.005$; GT: $p = 0.015$) (Figure IV.4A). Accordingly, six genes were assembled on the same network associated to antigen presentation and immune response (Figure IV.4B).

The analysis of FGF19 treatment in S252W fibroblasts pointed to 46 DEGS (Table IV.1- Anexo), where, in addition to immune cell function (IPA: $p = 0.001$; DAVID: $p = 0.03$; GT: $p = 0.001$), there was an enrichment for genes related to cell proliferation (IPA: $p = 0.001$; GT = 0.03) and ossification (IPA: $p = 0.0006$; GT $p = 0.006$) (Figure IV.4C). Ten of the DEGS were assigned to the same network associated to antimicrobial response and inflammation (Figure IV.4D).

Figure IV.1. Over-(red) or under-represented (green) biological categories and pathways among DEGs in FGF2 treated WT fibroblasts (GeneTrail).

A

Figure IV.2. (A) Gene network associated with cell movement and cell proliferation found among DEGs in FGF2 treated WT fibroblasts (IPA). (B) over-(red) or under-represented (green) biological categories and pathways among degs in FGF2 treated S252W fibroblasts (Genetrail). (C) Gene network associated with cell movement and cell proliferation found among DEGs in FGF2 treated S252W fibroblasts (IPA) (red: up-regulated DEG, green: down regulated DEG)

Figure IV.3. (A) OVER-(red) or under-represented (green) biological categories and pathways among DEGs in FGF10 treated S252W fibroblasts (GeneTrail). (B) Gene network associated with cell movement and cell proliferation found among DEGs in FGF10 treated S252W fibroblasts (IPA) (red: up-regulated DEG, green: down regulated DEG).

Figure IV.4. (A) Over-(red) or under-represented (green) biological categories and pathways among DEGs in FGF19 treated WT fibroblasts (GeneTrail). (B) Gene network associated with cell movement and cell proliferation found among DEGs in FGF19 treated WT fibroblasts (IPA) (red: up-regulated DEG, green: down regulated DEG). (C) Over-(red) or under-represented (green) biological categories and pathways among DEGs in FGF19 treated S252W fibroblasts (GeneTrail). (D) Gene network associated with cell movement and cell proliferation found among DEGs in FGF19 treated S252W fibroblasts (IPA) (red: up-regulated DEG, green: down regulated DEG).

Validation of global gene expression analysis

To validate the gene expression microarray analysis, we conducted qRT-PCR of some DEGS identified between FGF treatments and control groups. The mRNA expression of *BDP1*, *CYP51A1*, *DUSP6*, *MAP4K4* and *STC1* were tested in WT fibroblasts treated with FGF2; *ARL17* in WT fibroblasts treated with FGF10; *IFI6* and *ARL17* in WT fibroblasts treated with FGF19, *BAT3*, *BDP1*, *CYP51A1* and *RFC3* in S252W fibroblasts treated with FGF2; *CFHR1*, *CLIC2*, *FAM60A* and *MGP* in S252W fibroblasts treated with FGF10; and *SNORA23*, *CCDC146*, *PARP14*, *IFI44* and *CKS2* in S252W fibroblasts treated with FGF19. The differential expression of each gene between treatment and control groups was calculated also as a fold-change value, and the correlation between these qRT-PCR fold-change and microarray analysis fold-change for each gene in each cell was evaluated by Spearman correlation test (Figure IV.5A). The correlation between the values of the two analysis in all cell lines and treatment groups was statistically significant ($r = 0.8$, $p < 0.001$). In conclusion, the values obtained from microarrays and qRT-PCR are consistent and therefore we believe that the DEGS selected by bioinformatics analysis are representative of transcriptome experiments.

Interestingly, *TCF19*, one common DEG between FGF2 and FGF10 treatment in S252W fibroblast (2.7 and 3.9 fold-change respectively) was also earlier found as one of the most upregulated genes in the specific gene expression signature our group reported (2.3 fold-change) (Fanganiello et al., 2007). Therefore, we have chosen also examine the protein levels of *TCF19* through immunofluorescence staining in two S252W fibroblasts not included in the microarray experiment. *TCF19* was only detectable when S252W fibroblasts were treated with FGF2 or FGF10. Results are in agreement with global expression investigation, further validating statistical analysis used in Affymetrix experiment (Figure IV.5B).

Investigation of CNS related genes in Apert mouse model

Neuroanatomical abnormalities are a striking phenotype that is part of the wide range of complications that characterize Apert syndrome and are much more severe than the ones present in other *FGFR2*-associated craniosynostosis (Raybaud & DiRocco, 2007). These anomalies are also present in *Fgfr2*^{+/S252W} mouse model at P0, and did not correlate with patterns of suture closure, suggesting that these alterations are a primary consequence of the mutation (Aldridge et al., 2010). Remarkably, almost 13% of the DEGs in FGF2 treated S252W fibroblasts were associated to neurological diseases.

Figure IV.5. (A) Validation of differentially expressed genes showing the correlation between fold-change obtained from the Affymetrix microarray experiment and the fold change values for each gene in each cell line and each treatment (FGF2, FGF10 and FGF19). The correlations between the values of microarray and qRT-PCR fold-changes were calculated through Spearman correlation test. (B) Immunofluorescence staining of TCF19 (green) in two lineages of S252W fibroblasts not included in microarray experiment after 24h treatment with PBS (control), FGF2 and FGF10. Blue staining refers to DAPI, amplification: 10x. (C) Correlation between mRNA levels of *BAT3*, *BDP1*, *CYP51A1*, *RFC3* and *TCF19* in C342Y fibroblasts and S252W fibroblasts.

Hence we evaluated the expression of these genes homologues in P0 *Fgfr2*^{+/S252W} mice whole brain (Wang et al., 2005) (Figure IV.6A, B, D, E, G, H and J) together with mRNA levels of the mutant receptor, *Fgfr2*, the epithelial isoform, *Fgfr2b*, the mesenchymal isoform, *Fgfr2c*, and of the *Fgf2* ligand gene (Figure IV.6C, E, I and K). We observed after analysis of the 7 genes through qRT-PCR that only *Strc* had differential expression in newborn Apert mice brain with a fold-change of 1.6 ($p=0.006$) (Figure IV.6J).

Gene expression analysis in cells heterozygous for a different *FGFR2* mutation

To delineate whether these transcriptional circuitry modifications were consequence of altered ligand binding affinity of *FGFR2* or of excessive intracellular signaling by the receptor, we sought for *BAT3*, *BDP1*, *CYP51A1*, *TCF19* and *RFC3* (DEGs of S252W fibroblasts in response to

Figure IV.6. Quantitative RT-PCR results for CNS related DEGs – *Bat3* (A), *H28* (B), *Hs6st1* (D), *Rfc3* (E), *Rps9* (F), *Tcf19* (G) and *Strc* (J) – as well as the receptor *Fgfr2* gene (C), the epithelial (F) and mesenchymal (I) isoform of the receptor and the ligand *Fgf2* (K), in p0 *Fgfr2*^{+/+} (WT) and *Fgfr2*^{+/S252W} littermates whole brain RNA.

FGF2) expression levels in a C342Y fibroblast through qRT-PCR. C342Y mutation in *FGFR2* causes Crouzon syndrome, which has some clinical features similar to those found in Apert syndrome, such as coronal synostosis and hypoplasia of the middle third of the face. However, C342Y mutation causes constitutive activation, i.e., there is an FGF-independent induction of FGFR2 signaling. The correlation of expression values between C342Y fibroblasts and S252W fibroblasts showed no correlation ($r^2=0.04$, $p=0.904$) (Figure IV.5C).

Discussion

Previously, we have shown that the S252W mutation leads to a more drastic function change in fibroblast than in mesenchymal stem cells taken from the periosteum of the coronal suture of AS patients (Fanganiello et al., 2007; Yeh et al 2011). In order to better understand the complexity of the AS phenotype, we chose to study the effect of FGFs 2, -10 and -19 (identified as the FGFs that alter cell proliferation of fibroblasts the most) on the transcriptome of the same cells.

We found that WT fibroblasts stimulated by FGF2 activated the transcription of genes involved in cell proliferation and migration, most particularly those involved with the activation of MAPK and JAK-STAT signaling pathways, thus consistent with the extensive literature in this field (Basilico & Moscatelli, 1992; Legeai-Mallet et al., 1998; Sahni et al., 1999; Boilly et al., 2000; Hart et al., 2000). Since S252W fibroblasts presents a unique gene signature of almost 300 DEGS (Fanganiello et al., 2007) which indicates a previously much altered gene expression profile, it is not surprising that treatment with FGF2 has a much greater effect on WT fibroblasts than on S252W fibroblasts. Importantly, even though under the same treatment, cells that have the receptor with increased affinity to the ligand is capable of carrying a different response. FGF2 induced differential expression of genes important for development and maintenance of the CNS only in AS fibroblasts. This finding is not surprising since the most abundant and widely distributed FGF in the central nervous system is FGF2 (Eckenstein et al., 1991; Zechel et al., 2010). It is localized in neurons and glial cells and is expressed in the CNS both during development and postnatally (Emoto et al., 1989; Chadi et al., 1993; Eckenstein, 1994). Of these genes, two – *BAT3* and *RFC3* – were validated through qRT-PCR, highlighting the importance of FGFR2 activation by FGF2 in this system. RFC3 is involved in the elongation process of primed DNA templates by DNA polymerase delta and DNA polymerase and a SNP in this gene was recently associated with bipolar disorder in a genome-wide association study (Baum et al., 2008). *BAT3* encodes a nuclear protein that is cleaved by caspase 3 and is implicated in the control of apop-

tosis and SNPs in this gene were associated to Alzheimer disease and multiple sclerosis in a genome-wide association study (Hafler et al., 2007; Li et al., 2008).

Although there is a list of DEGs due to FGF10 treatment in WT fibroblasts, no significant functional enrichment was found, suggesting that this ligand does not transactivate FGFR2. The high local concentrations of FGF10, expressed in mesenchymal origin tissues, could allow an autocrine signaling through the pathological activation of mutant FGFR2, once it was demonstrated that binding of FGF10 to FGFR2c^{S252W} occurs on biochemical tests (Ibrahimi et al., 2004) and that FGF10 stimulates murine cells expressing FGFR2c^{S252W}, but not in the same cells expressing the wild-type FGFR2c (Yu et al., 2000). Our results suggest that this pathological effect occurs through an increase in the signaling pathways involved in immune response. In S252W fibroblasts, the most important signaling pathways induced by FGF10 do not coincide with those regulated by the induction of FGF2, with little intersection between those two DEGs lists. One of the genes with increased expression was *MGP* (Matrix Gla-Protein; Fold-Change = 1.94), validated by qRT-PCR. Mutations in this gene result in a nonfunctional protein and causes Keutel syndrome, a rare genetic disorder characterized by pulmonary stenosis, and calcification of cartilage brachytelephalangism (Munroe et al., 1999).

Another remarkable result of this treatment was the differential regulation (up-regulation of *HLA-DMA*, *RING1*, *ORD12D3*, *TCF19*, *CLIC2*, *LY6G5* and *MOG* and down-regulation of *ALPP*, *KRT18*, *XCL1* and *C6ORF15*) of several genes involved in inflammatory diseases. Of these genes, we validated *CLIC2* through qRT-PCR. Increased expression of the gene encoding this chloride channel in leukocytes is associated with systemic juvenile idiopathic arthritis (Allantaz et al., 2007). Wound healing in adult humans is a dynamic process that consists of precisely arranged events, it begins with quick hemostasis and appropriate inflammation, followed by cell proliferation and migration into the wound site, cell differentiation and suitable angiogenesis (Gosain & DiPietro, 2004; Allantaz et al., 2007). Since the recurrence of fusion of the sutures after craniotomy in patients with Apert syndrome depends on the rapid healing, our data also suggest a role of FGF10 in fibroblasts S252W as important in this process.

Among the two FGF treatment discussed, one interesting common DEG was *TCF19*, given that it was highly expressed in S252W fibroblasts (Fanganiello et al., 2007). The higher expression of *TCF19* was also confirmed by immunohistochemistry. Although *TCF19* has been considered susceptibility locus for some CNS and immunological diseases (Teraoka et al., 2000; Nair et al., 2006; Hafler et al., 2007), little is known about its function. Ku and colleagues (Ku et al., 1991) reported that mRNA levels of *TCF19* could only be detected after serum stimulation of beforehand-starved 3T3

cells. In view of the fact that in our previous study *TCF19* was upregulated when S252W fibroblasts were compared to WT fibroblast in fetal bovine serum rich medium (Fanganiello et al., 2007) and that *TCF19* protein was only detected in FGF2 or FGF10 treated S252W fibroblasts but not in the absence of FGFs, we conclude that important factors in the serum that activates the expression of *TCF19* are these two ligands. Nevertheless, it would be important to determine if *TCF19* has a significant role in Apert syndrome phenotype.

Although it has been demonstrated by Ibrahimi et al. (Ibrahimi et al., 2004) that S252W mutation has a strong effect on binding of FGF19 to FGFR2c, a possible connection between FGF19 and Apert syndrome has not been considered. In the presence of the S252W mutation, FGF19 is able to bind to FGFR2 and this activation may be responsible for altering the expression of genes associated to cell proliferation and ossification as we observed previously. Since this is an atypical FGF that acts as a hormone and is found in the bloodstream, it would be interesting to look at the transcription network resultant from the activation of FGFR2c^{S252W} by this FGF. It was demonstrated in different cell lines (Hela, HEK293 and DU145) that stimulation of FGFR4 by FGF19 leads to inhibition of the NFκβ, involved in inflammatory response (Drafahl et al., 2010). Therefore, the enrichment of genes associated with inflammatory/immune response in both WT and mutant fibroblasts might be consequence of the activation of FGFR4 by FGF19.

Megaencephaly and benign distortion ventriculomegaly are landmarks of Apert syndrome (Cohen & Kreiborg, 1990, 1994; Cohen, 2000; Renier et al., 2000; Yacubian-Fernandes et al., 2004). Other common CNS alterations observed in Apert syndrome patients are agenesis of the corpus callosum (Cohen & Kreiborg, 1990, 1991a, 1994), anomalies in limbic structure (de Leon et al., 1987; Cohen & Kreiborg, 1990, 1991a; Renier et al., 2000; Quintero-Rivera et al., 2006), and in gyral patterning (Cohen & Kreiborg, 1990, 1991a). Although brain size is not increased in *Fgfr2*^{+/S252W} mouse model at P0, other CNS anomalies in these animal were found to be highly correlated to the human phenotype (Aldridge et al., 2010). However, molecular signaling that links FGFR2 mutation to these malformations remains unclear. In Apert mouse brain, there was no differential expression of *Fgfr2* or *Fgf2*, neither of 6 out of the 7 CNS associated genes tested. Species-specific and tissue-specific differences might account for why most DEGs were not validated by this system. Also, it is known that the brain continues to develop throughout infancy until adulthood, so that these DEGs might present differential expression in other period.

We found upregulation of Stereocilin, consistent with S252W fibroblast microarray analysis (Table IV.1). Loss of function mutations in human *STRC* gene are causative of autosomal recessive

deafness (Verpy et al., 2001) and *Strc* knockout mice also show hearing impairment (Verpy et al., 2008). Though it is also expressed in the brain, eyes, testis and lungs, the role for stereocilin is better established in sensorial hair cells in the cochlea (Verpy et al., 2001). It is localized at the apical end of kinocilium, primary cilia of hair cells, and is thought to be responsible for the establishment of interaction between stereocilia (specialized motile cilia) and tecta membrane (Verpy et al., 2010). While *Strc* was not studied in neurons, we know that primary cilia is important for the adequate development of CNS and that the so called ciliopathies show not only craniofacial anomalies but also severe CNS malformations (Brugmann et al., 2010; Goetz & Anderson, 2010). Moreover, it is known that FGF signaling regulates the proper formation of primary cilia (Neugebauer et al., 2009; Goetz & Anderson, 2010). *Strc* was found to be differentially expressed in Apert mice brain at the P0 stage, but not in E16 (data not shown), which indicates that abnormal upregulation of stereocilin mRNA is rather a consequence of altered *Fgfr2* signaling in CNS development, than a cause of these malformations.

Additionally, expression of FGF2 treatment DEGs in *FGFR2*^{+/C342Y} fibroblasts did not correlate with the expression levels obtained in *FGFR2*^{+/S252W} fibroblasts. C342Y mutation in *FGFR2* (Crouzon syndrome) leads to a ligand- independent activation of the receptor (Mangasarian et al., 1997), while S252W mutation in *FGFR2* (Apert syndrome) leads to an unspecific ligand affinity of the receptor (Yu et al., 2000; Ibrahimi et al., 2004). However, Crouzon patients have milder phenotype compared to Apert individuals, which indicates that these two mutations have different molecular and cellular consequences. Our results confirm that two different types of gain-of-function mutation result in distinct transcriptional signaling in the same cell type and in the presence of a same ligand.

We not only corroborated previous data on FGF2 treatment in WT fibroblasts, but also revealed different pathways activated by this same treatment in the S252W fibroblast, and showed that different FGFs may trigger different downstream signaling pathways in cells expressing mutant *FGFR2*. Some of the abnormal signals induced by FGF2 may be linked to some of the central nervous system changes observed in patients with Apert syndrome. The exposure of S252W fibroblasts to FGF10 shows for the first time an involvement of pathways that may help explain the recurrent synostosis AS. Our expression data, as well as our previous results of osteogenic differentiation, suggest for the first time an involvement of FGF19 in bone formation in Apert cells. Furthermore, qRT-PCR analysis of C342Y fibroblasts demonstrated that fibroblasts that harbor promiscuous *FGFR2* have a distinct signaling and gene expression profile from cells with merely exaggerated activation of the receptor. Thus, gene expression profile delineated in the present study is representative of Apert syndrome and Apert mouse model study suggests a role for altered primary cilia in Apert CNS malformations.

Materials and methods

Subjects

Coronal suture periosteal fibroblasts from three AS patients (FGFR2^{+/S252W}) and from three age- and sex-matched control subjects were obtained as described earlier by us (Fanganiello et al., 2007). Fibroblasts from the same region from a Crouzon syndrome patient were obtained following the same protocol. The presence of the S252W and the C342Y mutation was confirmed by direct DNA sequencing and expression of FGFR2c in the primary fibroblasts was examined by Western Blot and RT-PCR.

Cell culture

Primary periosteal fibroblasts derived from periosteal flaps were grown in fibroblast growth medium (DMEM High-Glucose, 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 2 mmol/L l-glutamine, penicillin, and streptomycin), in a humidified incubator at 37°C and 5% CO₂. Cells were passaged at near confluency with trypsin-EDTA. All tests were performed between the third and the fifth subculture.

Exogenous FGF treatment

Periosteal fibroblasts were grown until they reached 80% of confluency. Cells were washed with PBS and then were serum starved for 24h in DMEM not supplemented with FBS. After this period control cells were treated with DMEM High-Glucose, 0.5% FBS and experimental cells were treated with DMEM High-Glucose, 0.5% FBS supplemented with recombinant human FGF2 or FGF10 (PeproTech, Rocky Hill, NJ, USA – diluted in PBS 1X to a final concentration of 2000 pM) or with DMEM High-Glucose, 0.5% FBS supplemented with PBS 1x free of FGFs. When treated with 2000 pM of FGFs (Yu et al., 2000), it was reported similar phosphorylation of both wild type and FGFR2c^{S252W}. Control and experimental cells were harvested at 24h after addition of FGFs, and had its total RNA isolated and purified as described below. Besides the biological triplicate, to account individual variability, the experiments were performed in technical triplicate for each sample, in order to consider possible experimental variability.

Generation of mutant mice

The Apert $Fgfr2^{+/S252W}$ mice were generated in the laboratory of Dr. Ethylin Wang Jabs (Wang et al., 2005). They were consistently inbred to a C57BL/6J background to minimize phenotypic variation due to genetic differences. Genotyping of tail DNA to distinguish mutant from wild-type progeny was carried out by polymerase chain reaction analysis. The primers for *Fgfr2* were as described (Wang et al., 2005). Care and use of mice for this study were in compliance with the relevant animal welfare guidelines approved by the Johns Hopkins University Animal Care and Use Committee and the Mount Sinai School of Medicine Animal Care and Use Committee. Mice were killed on P0 by inhalation anesthetics and weighed. The carcasses were fixed and whole brains were perfused in RNA later. Our sample consists of two litters inbred in different time, each consisting of two $Fgfr2^{+/S252W}$ and six wild-type littermates.

RNA extraction

Cells at a confluency of 80% in 25 cm² cell culture bottles were used for FGF treatment for microarray and qRT-PCR assays. After a 24h starvation period AS and WT cells were treated with DMEM High-Glucose without FBS supplemented with recombinant human FGF2 or FGF10 or with DMEM High-Glucose, without FBS supplemented with 1xPBS free of FGFs. Total RNA was isolated from FGF treated and untreated cells using Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) for 24h (when we first verified the expression level of genes upregulated by $FGFR2^{+/S252W}$, similar significant alterations in these genes in FGF2 induced control fibroblasts was only observed after 24h (Fanganiello et al., 2007)).

Mice whole brain RNA was extracted with RNeasy Mini Kit (Qiagen) following manufacturer's instruction.

RNA quality and concentration were accessed by 1.5 percent agarose gel electrophoresis and Nanodrop ND-1000 (Thermo Scientific, Waltham, Massachusetts, USA) respectively.

Microarray Assays

For each cell line, cDNA was generated with the Affymetrix GeneChip WT cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, California) following the manufacturer's instruc-

tions. cDNA was fragmented and end labeled with the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, California). Approximately 5.5 µg of labeled DNA target was hybridized to the Affymetrix GeneChip Human Gene1.0 ST array (Affymetrix, Santa Clara, California) (which interrogates 28869 well-annotated genes) at 45°C for 16h per manufacturer's recommendation. Hybridized arrays were washed and stained on an Affymetrix GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, California) and scanned on an Affymetrix GCS 3000 (Affymetrix, Santa Clara, California).

Intensity data were subjected to Robust Multichip Average (RMA) and afterwards, to identify differentially expressed genes (DEGs), we used the Limma (Wettenhall & Smyth, 2004) and RankProd (Hong et al., 2006) methods, available in the R/Bioconductor package, both with p-value ≤ 0.05 adjusted by FDR (False Discovery Rate) correction factor.

In order to minimize biological variations and focus on the effect of the ligand, we compared the expression data of all three treated cell populations, whether WT or FGFR2^{+/S252W}, with the corresponding expression data of the same three untreated cell populations. We extracted the genes that were commonly selected by two different methods (RankProd and Limma) as significantly differentially expressed (DEGS) in order to minimize false positive occurrence. The Limma method performs statistical analysis similar to that used by SAM (Significance Analysis of Microarrays) (Tusher et al., 2001), and is based on a moderate t-statistics to test the average difference in log expression levels between the treated and the control groups for each gene. The RankProd is a rank-based non-parametric method that uses geometric mean rank for each gene and its distribution is estimated by randomly permuting the observed ranks. The permutation principle partly alleviates the small sample sizes issue, enhancing the robustness against outliers (Saeys et al., 2007). To analyze the result, we used the IPA software for the analysis of gene interaction and functional classification of DEGS; DAVID for the enrichment of gene ontology and GT (GeneTrail) for analysis of over-or under representation of biological categories and pathways.

Reverse Transcription Reactions and Quantitative Real Time PCR

Complementary DNA (cDNA) was produced from 1 µg of total RNA using Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA, USA).

For the fibroblast qRT-PCR, assay was performed using approximately 20 ng of cDNA and SYBR Green PCR master mix in an ABI Prism 7500 system (Applied Biosystems, California, USA).

For mouse brain qRT-PCR, experiments were run with 20 ng of cDNA and SYBR Green PCR master mix in an ABI Prism 7900 system (Applied Biosystems, California, USA). The PCR conditions for both were: 95°C for 15s, 60°C for 30s, and 72°C for 30s for 40 cycles. In the mouse brain study, first it was performed in a paired 2 WT: 2 S252W littermates sample, if significant difference was observed, sample size was increased to 12 WT: 4 S252W from two litters.

Primers were designed with Primer Express software V.2.0 (Applied Biosystems, California, USA) and the amplification efficiency (E) of each primer was calculated according to the equation $E=10^{(-1/\text{slope})}$. The expression data of the studied transcripts was determined by relative quantification in comparison to four endogenous controls (*GAPDH*, *HMBS*, *HPRT1* and *SDHA*). We verified the gene expression stability of endogenous controls through geNorm VBA applet designed for Microsoft Excel. This tool calculates the most stable reference genes from a set of tested candidate reference genes in a given sample panel, and calculates the gene expression normalization factor for each target sample based on the geometric mean of a defined number of housekeeping genes (Vandesompele et al., 2002). The expression data is given by the ratio between each transcript $\Delta\Delta\text{Ct}$ ($E^{\Delta\Delta\text{Ct}}$) and a normalization factor. Samples from all cells analyzed previously in Microarray assay were run in technical triplicates, and the threshold suggested by the instrument software was used to calculate Ct. Primers used in this study are summarized in Table 2.

To assess the statistical significance of the correlation between microarray assay data and the qRT-PCR results we used the nonparametric two-tailed Spearman correlation test, with p-values of less than 0.05 considered to be statistically significant.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized in 0.05% Triton X-100 in PBS for 5 min. Nonspecific binding was blocked with 10% BSA in PBS for 1h at room temperature. Cells were incubated with primary antibody against TCF19 (1:100, Sigma) overnight at 4°C. After several washes, cells were incubated with secondary (1:100, AlexaFluor 488, Invitrogen) antibodies against mouse IgG tagged with for 2h at room temperature. Slides were counterstained with DAPI (4'-6-diamidino-2-phenylindole, Sigma). All images in the same set (treatments and controls) were obtained using the same photographic parameters of exposition and speed. Images were captured using the Axiovision 3.0 image analysis system (Carl Zeiss).

Table IV.2. Primers used for quantitative real time PCR.

Gene	Primer Forward	Primer Reverse
<i>ARL17</i>	AGTCATCCTTTCTCCCCAT	CCAGAGGATGGAAGAAGTCAGG
<i>BAT3</i>	GGAAGTATCGCTTCGAGATCCC	TATCATTAGGCTCCATGGCCG
<i>BDP1</i>	CTAACAAGCTGTCCACAACCG	CGAACTCGTTTTGATCCACG
<i>CCDC146</i>	TGACGCCGTGATGAGCACACAA	TGGAGACCTCCGTGGAGAATGCTT
<i>CDH1</i>	AGGAGAGCGGTGGTCAAAGAGC	CAGCTGGCTCAAGTCAAAGTCCTG
<i>CFHR1</i>	CCACCTCAATGCAAAGATTCTACG	GCATTGGTACTCAACTGATGAAGC
<i>CKS2</i>	CGAGTACCGGCATGTTATGTTACC	AACCCAGCCTAGACTCTGTTGG
<i>CLIC2</i>	CTGATTGTAGCTTGTTACCCAAGC	CATAGGCATTGTGGAGATAACGC
<i>CYP51A1</i>	GCCATTGTTTGCATGGAAAGG	AACTAGGCAAAGGCAGCCAACC
<i>DDX58</i>	TACATCTGAGCTACATGGCCC	AGAAAAAGTGTGGCAGCCTCC
<i>DUSP6</i>	GCCGCAGGAGCTATACGAGT	CCGTATTCTCGTTCAGTCG
<i>FAM60A</i>	GTTTTGGATTGCATGAGACTCG	CCTTGCATCTACCACATGATTCC
<i>FSP1</i>	CTCTACAACCTCTCTCTCAGCG	CCTTCTCCAGAGGGCAGCCAT
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATG
<i>HLA-DMA</i>	AGGTGGTACTGATTCTCCAGACC	CCCAGAGACTTCTACCCTAAGAGG
<i>HMBS</i>	GGCAATGCGGCTGCAA	GGGTACCCACGGAATCAC
<i>HPRT1</i>	TGACACTGGCAAAACAATGC	GTCCTTTTACCCAGCAAGC
<i>IFI44</i>	TCCCCATCGCTGAAGGACAGAA	GCCACATGTACCACACCAGCGT
<i>IFI6</i>	CCTCCAAGGTCTAGTGACGGAGCC	TGCCTCCACCCCACTGCAAG
<i>MAP4K4</i>	AGCATCATACTGGAAAGCAAACA	AAACCAACGGCATGTTTCATCGT
<i>MGP</i>	TCACAGCCTTCCACTAACATCCC	AGGATGGCAAGAAGGATCAGG
<i>NOG</i>	AGCGCCTAAGCAAGAAGCTGCG	CTTACGTAGCGCGGCCAAA
<i>OAS3</i>	TCATTTGGTACTGGCTACCTGG	TGTACCACCACTTTCCTACTGC
<i>PARP14</i>	TCCAGAGCCCGAAGAGGTCG	TGCCGAACGTCTCCGGGTAG
<i>RFC3</i>	ATGGCAGCTTACTATGAGCATCG	TCATGCCTTCCAATCCATCC
<i>SAMHD1</i>	AGGATGTCTAGTTCACGCACTGG	TGAGAAAATGGCCCATGACC
<i>SDHA</i>	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
<i>SNORA23</i>	GCTATCCACACAAACATCATGCGGC	AATTGGAGGCTGGCCAGTGGT
<i>STC1</i>	TCAACAGTGCTCTACAGGTCG	CCTGAGTGTCAAATTTAGCAGC
<i>Bat3</i>	TGATCTGCGCTGCAATCTAGCC	TGCTGTGATGGTCACAGTAGTCC
<i>Fgf2</i>	CCAACCGGTACCTTGCTATGA	TTCCGTGACCGGTAAGTATTGTAG
<i>Fgfr2</i>	CGAATGAAGACCACGACCAA	GACTCGGCCGAACTGTTACC
<i>Fgfr2b</i>	ACGTGGAAAAGAACGGCAGTAA	AGAGCCAGCACTTCTGCATTG
<i>Fgfr2c</i>	AGTGGATCAAGCACGTGGAAA	CCTCAATCTCTTTGTCCGTGGT
<i>H28</i>	TCCGGGTCACAGCATCAGTTACAG	TGCGTTCCCAAGCAGCTTTTCC
<i>Hs6st1</i>	TCTGCGTTCGCCCAGAAAGTTC	ATGTGCAAGGAGGTCTTCCACGTG
<i>Rfc3</i>	ATCCACTGTCTGCAGGAAGGAAGG	AAGGGTATTGCTGCACTCTGCAGG
<i>Rps9</i>	ATGGACTCCGGAACAAACGTGAGG	AGCATTGCCTTCAAACAGACGCC
<i>Strc</i>	TGGCTCTTTCAGCATTACTGCGGG	AAGACCACTGCAAACCTGGGAGC
<i>Tcf19</i>	TCACAGCAGCCAAGGGACTTTG	AGAACTCTGAGGAGGAGCTGGTTC

Statistical analysis

Statistical analysis was performed using the GraphPad InStat software (GraphPad). Continuous variables were expressed by mean and standard deviation and the groups were compared by Student's t-test. A p value < 0.05 was considered statistically significant.

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V. Complicações cirúrgicas em pacientes com Síndrome de Apert podem estar relacionadas com o tipo da mutação do paciente

Surgical complications in patients with Apert syndrome may be related to the type of mutation in the patient

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Abstract

Introduction: Apert syndrome is a severe form of craniosynostosis associated with syndactyly. Over 98% of the cases are caused by two mutations in the *FGFR2* gene. Here we evaluate the case of KS, a patient with atypical mutation (c.1119-2 A> G) that, unlike patients with typical mutations, developed extracapsular temporomandibular ankylosis after the third cranial surgery. This mutation leads to the unusual presence of ectopic epithelial isoform of *FGFR2* in mesenchymal cells. As it is well known that signaling by *FGFRs* can lead to formation of fibrosis through epithelial-mesenchymal transition (EMT), our goal was to determine whether fibrosis following the surgical procedure might have been predisposed by an atypical mutation in KS.

Methods: We compared the expression of the epithelial and mesenchymal isoforms of *FGFR2*, as well as a marker of EMT, collagen type I, in fibroblasts from Apert patients with typical mutation and controls without craniosynostosis compared to fibroblasts and mesenchymal stem cells of KS from the ankylosis and of a surgically unaffected region.

Results: The mutation c.1119-2A> G does not alter the overall levels of *FGFR2*, but increases the expression of the epithelial isoform. In the fibrotic region, both types of cells from patient KS have a dysregulation of the transcription process of the isoforms of *FGFR2*, with increased expression of both isoforms. Quantitative analysis of the expression of collagen type I showed that KS patient's fibroblasts express *COL1A1*, whether they came from the region of fibrosis or not; and that in mesenchymal stem cells from KS, this increase was only observed in cells from the fibrosis.

Conclusions: The results indicate that the surgical procedure stimulated fibrogenesis, through EMT, which can be induced by the mutation c.1119-2A> G. These data exemplify the reflection of genetic factors in determining surgical prognosis.

Key words: Apert syndrome, Epithelial-Mesenchymal Transition, Genotype

Resumo

Introdução: A síndrome de Apert é uma forma grave de craniossinostose associada à sindactilia. Mais de 98% dos casos são causados por duas mutações no gene *FGFR2*. Aqui avaliamos o caso da paciente KS com mutação atípica (c.1119-2 A>G) que, diferentemente de pacientes com mutações típicas, desenvolveu após a terceira cirurgia craniana, uma anquilose têmporo-mandibular extracapsular. Esta mutação atípica leva à presença ectópica da isoforma epitelial de *FGFR2* em células mesenquimais. Como é conhecido que a sinalização por FGFRs podem levar à formação de fibrose através da transição epitélio-mesenquimal (EMT), nosso objetivo foi verificar se reação exacerbada em relação ao processo cirúrgico poderia ter sido predisposta pela mutação atípica em KS.

Métodos: Comparamos a expressão das isoformas epitelial e mesenquimal de *FGFR2*, além de um marcador de EMT, o colágeno I em fibroblastos de paciente Apert com mutação típica e de controles sem craniossinostose comparados com fibroblastos e células-tronco mesenquimal de KS da região da anquilose e de região não cirurgiada.

Resultados: A mutação c.1119-2 A>G não altera os níveis totais do *FGFR2*, mas aumenta a expressão da isoforma epitelial. Na região da fibrose, ambos os tipos celulares da paciente KS apresentam uma desregulação do processo de transcrição das isoformas de *FGFR2*, com aumento de expressão das duas isoformas. A análise quantitativa da expressão do colágeno I revelou que os fibroblastos da paciente KS expressam *COL1A1*, independente se foram oriundos da região da fibrose ou não; nas células-tronco mesenquimais de KS, o aumento só foi verificado nas células provenientes da fibrose.

Conclusões: Os resultados apontam que o procedimento cirúrgico estimulou fibrogênese, com a participação de EMT, o que pode ser induzido pela mutação c.1119-2 A>G. Estes dados exemplificam o reflexo de fatores genéticos na determinação do prognóstico cirúrgico.

Palavras-chave: Síndrome de Apert, Transição Epitelial-Mesenquimal, Genótipo

Introdução

A síndrome de Apert (SA) é uma doença genética grave de herança autossômica dominante que se caracteriza clinicamente pela craniossinostose (fusão prematura das suturas cranianas) e sindactilia óssea bilateral de mãos e pés (Cohen, 1975). Outras malformações menos frequentes podem estar presentes, como anormalidades de pele, esqueleto, cérebro e órgãos internos (Cohen & Kreiborg, 1993d; Yu et al., 2000). A reabilitação destes pacientes consiste em múltiplos procedimentos cirúrgicos nos primeiros 10 anos de vida, sendo que intervenções no crânio são imprescindíveis para impedir a compressão do cérebro e consequentes danos ao sistema nervoso central (Cohen, 2000).

Mais de 98% dos casos de SA são causados por dois tipos de mutações, p.Ser252Trp ou p.Pro253Arg, no gene *FGFR2*, o qual codifica o receptor do tipo 2 de fator de crescimento de fibroblasto (Wilkie et al., 1995). Outras craniossinostoses também são causadas por mutações em *FGFR2*, tais como Crouzon, Pfeiffer, Jackson-Weiss e Beare-Stevenson (Passos-Bueno et al., 1998).

Há duas isoformas principais da proteína *FGFR2*, uma que se expressa especificamente em tecido de origem epitelial (*FGFR2IIIb*) e outra que se expressa em tecido mesenquimal (*FGFR2IIIc*). Estas isoformas apresentam alta especificidade de ligação aos diferentes fatores de crescimento de fibroblastos (FGFs). Assim, além destas isoformas serem tecido-específicas, a ativação de cada uma delas depende da presença de determinados ligantes, de forma que há um controle preciso da ativação das isoformas de *FGFR2* durante o desenvolvimento embrionário e o período pós-natal.

As duas mutações clássicas associadas à SA ocasionam um ganho de função às isoformas, causando uma ativação constitutiva do receptor. Porém, estas mutações não interferem no processamento do RNA, isto é, cada isoforma é expressa corretamente nos tecidos esperados.

Anteriormente, reportamos uma paciente (KS) com quadro clínico compatível com o da SA, porém, portadora de outra mutação, a c.1119-2 A>G no gene *FGFR2* (Passos-Bueno et al., 1997). Esta mutação causa alteração do processamento do RNA do *FGFR2* em fibroblastos de pele, de forma que há uma maior produção da isoforma epitelial (Oldridge et al., 1999). Contudo, nunca foi verificado se esta mutação confere alteração do processamento de RNA em fibroblastos derivados de tecidos faciais ou em células tronco-mesenquimais. Cumpre ainda ressaltar que os efeitos clínicos desta mutação são muito pouco conhecidos e explorados, particularmente no período pós-natal.

A paciente KS foi submetida a três grandes cirurgias cranianas: duas intracranianas (avanço fronto-orbitário bilateral) e uma extracraniana (osteotomia tipo Le fort 3). Após esta última cirurgia,

a paciente desenvolveu uma anquilose têmporo-mandibular extracapsular, o que provocou uma grave limitação da abertura da boca devido à presença de uma extensa fibrose no músculo temporal.

Fibrose é definida pelo crescimento excessivo, endurecimento, ou cicatrização de vários tecidos, e é atribuída à deposição excessiva de componentes da matriz extracelular, incluindo o colágeno do tipo 1 (Zeisberg & Neilson, 2009). Um dos processos envolvidos com a fibrose mediada pela sinalização FGF e FGFR é a transição epitélio-mesenquimal (EMT) (Strutz et al., 2002; Kalluri & Weinberg, 2009). Considerando-se a natureza da mutação presente na paciente KS, levantamos a hipótese de que esta reação exacerbada em relação ao processo cirúrgico poderia ter sido predisposta pela presença ectópica da isoforma epitelial de FGFR2 em células mesenquimais.

Com o objetivo de testarmos esta hipótese, verificamos: a) se a mutação c.1119-2 A>G propicia a produção da isoforma epitelial em células mesenquimais (fibroblastos e células-tronco mesenquimais) de tecidos craniofaciais; b) se há uma alteração do perfil de expressão das isoformas de FGFR2 nas células da região do tecido afetado, i.e. da região da fibrose, em relação as células de tecido não comprometido; c) se as células da região de fibrose apresentam maior produção de colágeno I, um marcador molecular do processo de fibrose e de EMT.

Resultados

a) Efeito da mutação c.1119-2 A>G nos níveis de expressão de FGFR2 e das isoformas epiteliais e mesenquimais

Para verificarmos se a mutação c.1119-2 A>G presente na paciente KS interfere nos níveis totais de *FGFR2* e no tipo de isoforma expressa, inicialmente comparamos os níveis de expressão de *FGFR2* em fibroblastos da região não afetada com os controles (Figura V.1).

Observamos que os níveis de expressão de *FGFR2* das células da paciente KS oriundas da região sem fibrose são semelhantes ao das células controles. Contudo, os níveis da isoforma mesenquimal estão reduzidos nas células da paciente KS em relação aos controles, enquanto os níveis da isoforma epitelial estão relativamente aumentados, sendo a razão de expressão entre as isoformas epitelial/mesenquimal de 13,3/0,38. Portanto, os níveis totais de *FGFR2* dos fibroblastos da paciente KS da região sem fibrose não diferem dos controles, porém expressam relativamente uma maior proporção da isoforma epitelial.

Figura V.1. Comparação da expressão relativa do *FGFR2* total entre as amostras da paciente KS oriundas da região não afetada pela fibrose (periósteo) e as amostras dos controles.

b) Efeito da fibrose nos níveis de FGFR2 e das isoformas epitelial e mesenquimal

Com o intuito de avaliarmos se há alteração do processo de transcrição de *FGFR2* nas células da região da fibrose, comparamos os níveis de expressão deste gene nas células da região da fibrose com as células da região não afetada.

Observamos que os níveis totais de *FGFR2* são mais elevados nas células da paciente KS oriundas da região da fibrose do que nas células da região não afetada, tanto nos fibroblastos (afetada/não afetada: 3.5/1.5) como nas células-tronco mesenquimais (afetada/não afetada: 2.3/1.2) (Figura V.2).

Figura V.2. Comparação da expressão relativa do *FGFR2* total entre os fibroblastos e as células tronco-mesenquimais oriundas de tecido da paciente KS com e sem fibrose.

Nos fibroblastos e nas células-tronco mesenquimais da região da fibrose, ocorreu um aumento expressivo das duas isoformas de *FGFR2* em relação às células da região não afetada (a isoforma epitelial teve um aumento de 9 vezes nos fibroblastos e de 5 vezes nas células-tronco mesenquimais (Figura V.3); e a isoforma mesenquimal teve um aumento de 11 vezes nos fibroblastos e de 5 vezes nas células tronco-mesenquimais (Figura V.4). Portanto, ambos os tipos celulares da região da fibrose apresentam uma desregulação do processo de transcrição das isoformas de *FGFR2*, com aumento de expressão das duas isoformas.

Figura V.3. Comparação entre a expressão relativa da isoforma epitelial do *FGFR2* entre as amostras da paciente KS oriundas das regiões afetadas e não-afetadas pela fibrose.

Figura V.4. Comparação da expressão relativa da isoforma mesenquimal do *FGFR2* entre as amostras da paciente KS oriundas das regiões afetadas e não-afetadas pela fibrose.

c) Expressão de Colágeno I

O colágeno 1 (*COL1A1*) é um dos produtos mesenquimais gerado pela EMT durante o desenvolvimento de fibrose em diversos órgãos, sendo utilizado como marcador deste processo. Com o intuito de verificarmos se a fibrose na paciente KS foi decorrente de EMT, avaliamos os níveis de *COL1A1* nas células da região afetada e não afetada da paciente KS bem como nos controles (Figura V.5).

A análise quantitativa da expressão do colágeno I revelou que os fibroblastos da paciente KS expressam *COL1A1*, independente se foram oriundos da região da fibrose ou não. Já nas células-tronco mesenquimais da paciente KS, somente a região da fibrose tem níveis expressivos de *COL1A1*, sendo que detectamos um aumento de aproximadamente 4,79 vezes de *COL1A1* nas células-tronco mesenquimais da região da fibrose quando comparadas com aquelas da região não afetada.

Figura V.5. Comparação entre a expressão relativa do Colágeno tipo I.

Discussão

A identificação de fatores responsáveis por complicações pós-cirúrgicas é extremamente relevante, pois pode-se adotar o uso de medidas preventivas que contribuam para uma melhor reabilitação dos pacientes, com um menor índice de complicações pós-cirúrgicas. Neste contexto, dada a complexidade das intervenções cirúrgicas nos pacientes com craniossinostose síndrômicas, o impacto da identificação e adoção de tais medidas pode ser bastante significativo.

A paciente KS representa o primeiro caso dentre mais de 25 pacientes com SA já operados pela equipe de cirurgia plástica do Hospital das Clínicas, USP (N.A. comunicação pessoal) em que ocorreu a produção de um excesso de fibrose no músculo temporal. Esta observação sugere, portanto, que esta complicação pós-cirúrgica não estaria associada a procedimento técnico e sim relacionado com a constituição genética da paciente.

Diferentemente de outros pacientes com SA, KS é portadora de outra mutação, a c.1119-2 A>G. Conforme observamos nos nossos experimentos, esta mutação, diferentemente da mutação típica de SA, a p.Ser252Trp, favorece preferencialmente a transcrição da isoforma epitelial em fibroblastos e células-tronco mesenquimais oriundas de tecido craniofacial. Portanto, os nossos resultados confirmam que a mutação c.1119-2 A > G altera o processamento do RNA (Passos-Bueno et al., 2008), e mostram que este efeito não é específico a fibroblastos de pele, mas também ocorre em células de origem craniofacial.

O início da indução da EMT é caracterizado pela substituição da isoforma epitelial de FGFR2 pela mesenquimal (Thiery & Sleeman, 2006; Shirakihara et al., 2011). No presente caso, constatamos um aumento da isoforma FGFR2IIIC nas células da região da fibrose e manutenção dos níveis de FGFR2IIIB, o que constitui uma evidência de que ocorreu indução do processo de EMT, já que fibroblastos originados de epitélio que sofreu fibrogênese ainda expressam os receptores e as interações moduladas de acordo com as células epiteliais das quais eles se originaram (Strutz et al., 1995).

Os maiores níveis de transcrito do COL1A1 detectados nos fibroblastos e células-tronco mesenquimais da região afetada corroboram a ocorrência de EMT, visto que o aumento de produção deste tipo de colágeno é um dos marcadores clássicos deste processo (Kalluri & Weinberg, 2009). A expressão ectópica da isoforma epitelial de FGFR2 nas células de KS pode ser um fator importante de predisposição a respostas celulares atípicas, tais como o processo de EMT após estímulo externo.

Em resumo, os resultados apontam que o procedimento cirúrgico estimulou fibrogênese, com a participação de EMT. Este processo pode ter sido mais facilmente induzido devido a presença da mutação c.1119-2 A>G, que causa uma expressão ectópica de FGFR2IIIB. Não podemos descartar, contudo, que a fibrogênese tenha sido decorrente da ativação de células mesenquimais residentes, e diferenciação fibroblasto-miofibroblasto ou de fibrócitos circulantes, as quais podem também ser responsáveis pela produção de COL1A1 (Wynn, 2008). Estes dados exemplificam o reflexo de fatores genéticos na determinação do prognóstico cirúrgico. Como ocorrido com a paciente KS, uma alteração genética pode exercer influência em um processo fisiológico aparentemente não relacionado ao quadro clínico principal, no caso o da SA, trazendo consequências durante seu tratamento. Desta maneira, uma vez melhor esclarecido o papel da mutação c.1119-2 A>G no gene FGFR2 sobre o mecanismo de fibrogênese, futuras intervenções cirúrgicas em pacientes portadores desta mutação poderão ser complementadas, por exemplo, com a aplicação de fármacos antifibrogênicos e/ou compostos direcionados ao bloqueio do processo de EMT.

Paciente e métodos

Obtenção de tecido e manutenção de culturas celulares

Durante correção cirúrgica da paciente KS foram obtidos tecidos da região afetada pela fibrose e periósteo de tecido nunca antes submetido a procedimento cirúrgico. Como controle para a análise, utilizamos periósteos cranianos que formam as suturas coronais de um paciente com a mutação mais frequente para a SA (p.Ser252Trp) e de um indivíduo controle (sem craniossinostose).

A obtenção de células de tecido e a manutenção das culturas seguiram protocolos definidos em nosso laboratório (Fanganiello et al., 2007; Passos-Bueno et al., 2008).

A obtenção do material foi realizada após consentimento do responsável conforme normas do Comitê de Ética em Pesquisa em Seres Humanos do Instituto de Biociências, USP (protocolo nº 024/2004).

Extração e amplificação de RNA

Após as células crescerem e a cultura atingir 80% de confluência em uma garrafa de cultura de 75 cm², as células foram lisadas e o RNA total extraído de acordo com o manual do kit de extração de RNA Nucleospin II (Macherey-Nagel).

O DNA complementar (cDNA) foi obtido pelo método de transcrição reversa (RT-PCR) a partir de 2 µg de RNA total utilizando o kit Superscript II (Invitrogen).

PCR em Tempo Real

O produto do RT-PCR é preparado para a reação de PCRq-TR utilizando o SYBR Green I (Applied Biosystems), que é um corante marcado com fluorescência verde que se liga ao DNA dupla-fita formado durante a reação de PCR.

As reações de PCRq-RT foram feitas no sistema de detecção Applied Biosystems 7500 (ABI, Foster City, CA, EUA) de acordo com o protocolo do fabricante do SYBR-Green Master Mix (idem). Os resultados foram analisados por meio do próprio programa 7500 Fast Real-Time PCR System.

As análises estatísticas foram realizadas através da comparação entre os valores da quantificação dos transcritos alvos e dos controles endógenos (genes envolvidos em funções básicas necessárias para a manutenção da célula): gene da hipoxantina-guanina-fosforribosiltransferase (*HPRT1*), da gliceraldeído-3-fosfato-desidrogenase (*GAPDH*), da hidroximetilbilano sintase (*HMBS*) e da subunidade A do complexo succinato desidrogenase (*SDHA*).

Os primers utilizados estão na tabela V.1 a seguir:

Nome do primer	Foward	Reverse
<i>FGFR2 total</i>	TCTGAGCCTTCGCAACTCGCGA	TGCCCTCGGATTGGGGAACGAGA
<i>FGFR2B</i>	ACCTCAAGGTTCTCAAGGCCGC	ACCCGCCAAGCACGTATATTCCC
<i>FGFR2C</i>	TCAATGTGACCGAGGCGGATGC	TCCAGGCGCTTGCTGTTTTGGC
<i>COL1A1</i>	CGAAGACATCCCACCAATCAC	CAGATCACGTCATCGCACAAC
<i>SDHA</i>	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
<i>GAPDH</i>	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATG
<i>HPRT1</i>	TGACACTGGCAAAACAATGC	GTCCTTTTACCCAGCAAGC
<i>HMBS</i>	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC

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VI. Discussão geral e conclusões

Por algum tempo o periósteo foi considerado apenas uma membrana limitante que envolvia os ossos do corpo. Atualmente, diversas evidências sugerem que o periósteo e a medula óssea são as principais fontes de células progenitoras com potencial osteogênico (Colnot, 2009). O estudo de fibroblastos e células-tronco mesenquimais (MSCs) extraídos do periósteo que recobre a sutura fusionada de pacientes de Síndrome de Apert é uma forma de entender a relevância deste tecido na patogênese desta síndrome, não só relacionado à fusão prematura das suturas coronais, mas também à reossificação dessas suturas mesmo após intervenção cirúrgica.

Apesar de não podermos distinguir estes dois tipos de células com base no imunofenótipo ou na morfologia celular, o estudo comparativo do fenótipo celular de fibroblastos e MSCs portadores da mutação S252W em FGFR2 nos mostrou o quão diferente são e nos permitiu delinear um modelo de como o periósteo influencia na fusão das suturas. A mutação S252W tem um efeito positivo sobre a proliferação e migração de fibroblastos, e um efeito negativo sobre a proliferação de MSCs e nenhum sobre a migração MSCs. Ambos os tipos celulares passam a apresentar maior diferenciação osteogênica e fibroblastos S252W têm influência positiva sobre a diferenciação MSCs. A inibição da fosforilação da JNK por SP600125 anula o efeito da mutação no processo de diferenciação osteogênica atípica de fibroblastos. A identificação da molécula-chave para a craniossinostose e associada à via de ERK-JNK é de grande importância, pois abriria perspectivas de desenvolver estratégias de terapia molecular associadas ao tratamento cirúrgico de pacientes com síndrome de Apert, de forma a contribuir com uma maior eficácia da reabilitação destes pacientes.

A análise do efeito dos ligantes sobre o receptor promíscuo adiciona mais detalhes ao modelo acima proposto. Nos fibroblastos S252W, os FGFs -2, -10 e -19 aumentaram a proliferação, o FGF2 aumentou a migração e o FGF19 desacelerou a diferenciação osteogênica. Nas MSCs S252W, o FGF2 aumentou a proliferação, o FGF10 aumentou a migração e o FGF19 aumentou a diferenciação osteogênica. O efeito osteogênico de FGF19 é uma descoberta interessante e indica possível relevância dos FGFs endócrinos para as várias anormalidades que estes pacientes apresentam pelo corpo todo, uma vez que são FGFs circulantes. Além disso, a resposta diferenciada da mesma célula para FGFs distintos aponta que, apesar do receptor com a mutação ser promíscuo, a forma como ocorre a interação do ligante por suas características intrínsecas com o receptor influenciam na sinalização intracelular ativada pelo FGFR2.

O estudo de alterações no transcriptoma derivado da ativação do FGFR2^{S252W} por estes FGFs em fibroblastos corroboram este último ponto. Também apontam para prováveis contribuições de cada FGF para a fisiopatologia da Síndrome de Apert. O FGF2 parece ter um efeito mais relevante às alterações do sistema nervoso central; o FGF10 estaria ligado à recorrente sinostose na síndrome; e FGF19, confirmando o nosso resultado anterior, participa na formação óssea em células de Apert. O estudo de expressão gênica no modelo murino para a síndrome de Apert sugere um papel do cílio primário nas malformações do SNC e comprova o estudo de fibroblasto como um modelo válido para esboçar o papel de outros tecidos nesta craniossinostose. Além disso, a comparação com fibroblastos também portadores de mutação em *FGFR2*, mas que altera as propriedades do receptor em um aspecto díspar ao do FGFR2^{S252W} corrobora que o perfil de expressão gênica delineado é representativo da síndrome de Apert.

Por último, o estudo de células de paciente com mutação rara para a síndrome de Apert mostra que apesar da expressão ectópica da isoforma epitelial em células mesenquimais gerar um fenótipo correspondente ao da Síndrome de Apert, a qual apresenta pouca variabilidade clínica, ela também leva a fenótipo atípico, neste caso, a fibrogênese. A expressão ectópica de FGFR2b parece ser responsável pela formação de fibrose através da transição epitélio-mesenquimal, o que não surpreende, uma vez que a via de FGF está associada a este processo.

Em conclusão, o presente estudo contribuiu para delinear a contribuição do periósteo e de diferentes FGFs para a Síndrome de Apert.

VII. Resumo

O crânio é composto de estruturas que interagem entre si formando um sistema complexo, como os ossos da caixa craniana unidos por tecido fibroso (sutura), o qual exerce função importante durante o desenvolvimento do indivíduo até a idade adulta. Ao fenômeno de fusão prematura das suturas dá-se o nome de craniossinostose que, em 32% dos casos com diagnóstico molecular, são causados por mutações no gene *FGFR2*. A via de sinalização por FGF já foi implicada tanto em processos biológicos mitogênicos, regulatórios, morfológicos quanto em processos endócrinos. A síndrome de Apert representa 4% de todos os casos de craniossinostose e as duas mutações mais frequentes encontradas nestes pacientes, S252W (64%) e P253R (26%), aumentam a afinidade de ligação dos receptores das isoformas epiteliais e mesenquimais do receptor por quase todos os FGFs e levam à perda de especificidade aos ligantes. Entretanto, a literatura acerca das características celulares aberrantes causadas por mutações desta síndrome é controversa. Atualmente, muitos estudos têm apontado a importância do periósteo, tecido fibroso rico em células que recobre os ossos, na regeneração óssea, não só através de sinalização parácrina, mas também como fonte de células osteoprogenitoras. Neste contexto, há poucos trabalhos na literatura.

Nossa hipótese principal é verificar se o periósteo contribui para a fusão, prematura e pós-cirúrgica, das suturas coronais na Síndrome de Apert. Neste caso, a nossa expectativa é as células que compõem este tecido, como por exemplo, fibroblastos e células-tronco mesenquimais, tenham funções celulares como proliferação, migração e diferenciação anômalas em resposta a vias de sinalização intracelulares alteradas. Assim sendo, nossos objetivos foram verificar se a mutação S252W tem um efeito funcional/celular semelhante em duas diferentes potenciais células osteoprogenitoras: fibroblasto e células-tronco mesenquimais; e verificar se diferentes ligantes a *FGFR2*, os FGFs, atuam diferentemente nas funções destas mesmas células com mutação S252W.

De forma geral, nossos resultados revelaram as diferenças funcionais entre fibroblastos e células-tronco mesenquimais (MSCs) provenientes de pacientes com síndrome de Apert, sendo que as funções dos fibroblastos mutados estão mais comprometidas do que as funções das MSCs. Além disso, os fibroblastos S252W têm efeito positivo sobre as MSCs, selvagem ou mutadas, enquanto que o oposto não ocorre. A inibição da fosforilação da JNK anula o efeito da mutação no processo de diferenciação osteogênica atípica de fibroblastos. Também mostramos que FGF2,

FGF10 e FGF19 têm diferentes influências sobre o fenótipo de células com a mutação, que também difere entre os tipos celulares. O FGF19 é o fator que mais interfere no processo de ossificação nas células S252W. Nossa análise de perfil de expressão gênica mostrou que os FGFs modulam diferentes vias de sinalização em fibroblastos de pacientes com síndrome de Apert: o FGF2 está ligado a genes do sistema nervoso central, corroborado pelo estudo no modelo animal; o FGF10, a resposta imune e o FGF19 à ossificação. O estudo de células com mutação atípica mostra que a expressão ectópica da isoforma epitelial de FGFR2 está associada ao fenótipo clínico da Síndrome de Apert e parece ser também responsável pelo fenótipo atípico associado à transição epitélio-mesenquimal. Estes resultados nos possibilitaram inferir que o perióstio contribui para o processo de reossificação das suturas na Síndrome de Apert, e que tanto fibroblastos como células-tronco mesenquimais podem estar envolvidos neste processo.

VIII. Abstract

The skull is composed of structures that interact with each other forming a complex system, such as the bones of the skull that are united by fibrous tissue (suture), which plays an important role during the development of the individual until adulthood. The phenomenon of premature fusion of sutures is named as “craniosynostosis”, which are caused by mutations in the *FGFR2* gene in 32% of the cases with molecular diagnosis. The FGF signaling pathway has been implicated in both mitogenic biological processes, regulatory, morphological and endocrine processes. Apert syndrome accounts for 4% of all cases of craniosynostosis and the two most frequent mutations found in these patients, S252W (64%) and P253R (26%), increase the binding affinity in mesenchymal and epithelial isoforms of the receptor for nearly all FGFs and lead to loss of ligand specificity. However, literature concerning the aberrant cellular characteristics caused by Apert syndrome mutations is controversial. Currently, many studies have highlighted the importance of the periosteum (a fibrous tissue rich in cells that covers the bones) in bone regeneration, not only through paracrine signaling, but also as a source of osteoprogenitor cells. In this regard, there are few studies in literature.

Our main hypothesis is to verify whether the periosteum contributes to premature and post-surgical fusion of the coronal sutures in Apert syndrome. In this case, we expect that the cells that compose this tissue, such as fibroblasts and mesenchymal stem cells have abnormal cell functions such as proliferation, migration and differentiation in response to altered intracellular signaling pathways. Our objectives were to verify if the S252W mutation has a similar functional/cellular effect in two different potential osteoprogenitor cells: fibroblasts and mesenchymal stem cells; and to verify whether different ligands to FGFR2, the FGFs, act differently in these cells with S252W mutation.

Overall, our results reveal functional differences between fibroblasts and mesenchymal stem cells (MSCs) from patients with Apert syndrome, and that these functions are more impaired in the mutant fibroblasts. Moreover, the S252W fibroblasts have a positive effect on the osteogenic differentiation of MSCs (wild-type and mutant) whereas the opposite does not occur. Inhibition of JNK phosphorylation nullifies the effect of atypical osteogenic differentiation of mutant fibroblasts. We also show that FGF2, FGF10 and FGF19 have different influences on the phenotype of mutant cells, which also differs between cell types. The FGF19 is the main factor that interferes with the process of ossification of S252W cells. Our analysis of gene expression profile showed that FGFs modulate different signaling pathways in fibroblasts from Apert syndrome patients: while FGF2 gene is linked to the central nervous system, supported by studies in animal model, FGF10 is associated to immune response and FGF19, to ossification. The study of cells with atypical mutation shows that the ectopic

expression of the epithelial isoform of FGFR2 generates the clinical phenotype of Apert syndrome, but also leads to an atypical phenotype associated with epithelial-mesenchymal transition. These results enabled us to infer that the periosteum contributes to the process of suture reossification in Apert syndrome, and that both fibroblast and mesenchymal stem cells may be involved in this process.

IX. Referências bibliográficas gerais

- Alberius, P., C. Dahlin, A. Linde (1992) Role of osteopromotion in experimental bone grafting to the skull: a study in adult rats using a membrane technique. *J Oral Maxillofac Surg* 50(8): 829-834.
- Aldridge, K., C.A. Hill, J.R. Austin, C. Percival, N. Martinez-Abadias, T. Neuberger, Y. Wang, E.W. Jabs, J.T. Richtsmeier (2010) Brain phenotypes in two FGFR2 mouse models for Apert syndrome. *Dev Dyn* 239(3): 987-997.
- Allantaz, F., D. Chaussabel, D. Stichweh, L. Bennett, W. Allman, A. Mejias, M. Ardura, W. Chung, E. Smith, C. Wise, K. Palucka, O. Ramilo, M. Punaro, J. Banchereau, V. Pascual (2007) Blood leukocyte microarrays to diagnose systemic onset juvenile idiopathic arthritis and follow the response to IL-1 blockade. *The Journal of experimental medicine* 204(9): 2131-2144.
- Allen, M.R., J.M. Hock, D.B. Burr (2004) Periosteum: biology, regulation, and response to osteoporosis therapies. *Bone* 35(5): 1003-1012.
- Anderson, J., H.D. Burns, P. Enriquez-Harris, A.O. Wilkie, J.K. Heath (1998) Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. *Human molecular genetics* 7(9): 1475-1483.
- Ang, B.U., R.M. Spivak, H.D. Nah, R.E. Kirschner (2010) Dura in the pathogenesis of syndromic craniosynostosis: fibroblast growth factor receptor 2 mutations in dural cells promote osteogenic proliferation and differentiation of osteoblasts. *The Journal of craniofacial surgery* 21(2): 462-467.
- Armelin, H.A. (1973) Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proceedings of the National Academy of Sciences of the United States of America* 70(9): 2702-2706.
- Artun, J., S.K. Osterberg, V.G. Kokich (1986) Long-term effect of thin interdental alveolar bone on periodontal health after orthodontic treatment. *J Periodontol* 57(6): 341-346.
- Aubin, J.E., A.K. Gupta, U. Bhargava, K. Turksen (1996) Expression and regulation of galectin 3 in rat osteoblastic cells. *Journal of cellular physiology* 169(3): 468-480.
- Bae, J.H., J. Schlessinger (2010) Asymmetric tyrosine kinase arrangements in activation or autophosphorylation of receptor tyrosine kinases. *Molecules and cells*.
- Baroni, T., P. Carinci, C. Lilli, C. Bellucci, M.C. Aisa, L. Scapoli, S. Volinia, F. Carinci, F. Pezzetti, M. Calvitti, A. Farina, C. Conte, M. Bodo (2005) P253R fibroblast growth factor receptor-2 mutation induces RUNX2 transcript variants and calvarial osteoblast differentiation. *Journal of cellular physiology* 202(2): 524-535.
- Basilico, C., D. Moscatelli (1992) The FGF family of growth factors and oncogenes. *Advances in cancer research* 59: 115-165.
- Baum, A.E., N. Akula, M. Cabanero, I. Cardona, W. Corona, B. Klemens, T.G. Schulze, S. Cichon, M. Rietschel, M.M. Nothen, A. Georgi, J. Schumacher, M. Schwarz, R. Abou Jamra, S. Hofels, P. Propping, J. Satagopan, S.D. Detera-Wadleigh, J. Hardy, F.J. McMahon (2008) A genome-wide association study implicates diacylglycerol kinase eta (DGKH) and several other genes in the etiology of bipolar disorder. *Molecular psychiatry* 13(2): 197-207.
- Beenken, A., M. Mohammadi (2009) The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov* 8(3): 235-253.
- Bialek, P., B. Kern, X. Yang, M. Schrock, D. Sasic, N. Hong, H. Wu, K. Yu, D.M. Ornitz, E.N. Olson, M.J. Justice, G. Karsenty (2004) A twist code determines the onset of osteoblast differentiation. *Dev Cell* 6(3): 423-435.
- Blank, C.E. (1960) Apert's syndrome (a type of acrocephalosyndactyly)-observations on a British series of thirty-nine cases. *Ann Hum Genet* 24: 151-164.
- Bochukova, E.G., T. Roscioli, D.J. Hedges, I.B. Taylor, D. Johnson, D.J. David, P.L. Deininger, A.O. Wilkie (2009) Rare mutations of FGFR2 causing apert syndrome: identification of the first partial gene deletion, and an Alu element insertion from a new subfamily. *Human mutation* 30(2): 204-211.
- Boilly, B., A.S. Vercoutter-Edouart, H. Hondermarck, V. Nurcombe, X. Le Bourhis (2000) FGF signals for cell proliferation and migration through different pathways. *Cytokine & growth factor reviews* 11(4): 295-302.
- Brugmann, S.A., D.R. Cordero, J.A. Helms (2010) Craniofacial ciliopathies: A new classification for craniofacial disorders. *Am J Med Genet A* 152A(12): 2995-3006.

- Bruner, E. (2007) Cranial shape and size variation in human evolution: structural and functional perspectives. *Childs Nerv Syst* 23(12): 1357-1365.
- Bueno, D.F., I. Kerkis, A.M. Costa, M.T. Martins, G.S. Kobayashi, E. Zucconi, R.D. Fanganiello, F.T. Salles, A.B. Almeida, C.E. do Amaral, N. Alonso, M.R. Passos-Bueno (2009) New source of muscle-derived stem cells with potential for alveolar bone reconstruction in cleft lip and/or palate patients. *Tissue Eng Part A* 15(2): 427-435.
- Burke, D., D. Wilkes, T.L. Blundell, S. Malcolm (1998) Fibroblast growth factor receptors: lessons from the genes. *Trends Biochem Sci* 23(2): 59-62.
- Cantley, L.C. (2002) The phosphoinositide 3-kinase pathway. *Science (New York, NY)* 296(5573): 1655-1657.
- Celil, A.B., P.G. Campbell (2005) BMP-2 and insulin-like growth factor-I mediate Osterix (Osx) expression in human mesenchymal stem cells via the MAPK and protein kinase D signaling pathways. *The Journal of biological chemistry* 280(36): 31353-31359.
- Chadi, G., A. Moller, L. Rosen, A.M. Janson, L.A. Agnati, M. Goldstein, S.O. Ogren, R.F. Pettersson, K. Fuxe (1993) Protective actions of human recombinant basic fibroblast growth factor on MPTP-lesioned nigrostriatal dopamine neurons after intraventricular infusion. *Experimental brain research Experimentelle Hirnforschung* 97(1): 145-158.
- Chan, W.Y., P.P. Tam (1988) A morphological and experimental study of the mesencephalic neural crest cells in the mouse embryo using wheat germ agglutinin-gold conjugate as the cell marker. *Development (Cambridge, England)* 102(2): 427-442.
- Chellaiah, A.T., D.G. McEwen, S. Werner, J. Xu, D.M. Ornitz (1994) Fibroblast growth factor receptor (FGFR) 3. Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. *The Journal of biological chemistry* 269(15): 11620-11627.
- Chen, L., D. Li, C. Li, A. Engel, C.X. Deng (2003) A Ser252Trp [corrected] substitution in mouse fibroblast growth factor receptor 2 (Fgfr2) results in craniosynostosis. *Bone* 33(2): 169-178.
- Chen, W., B. Obermayer-Pietsch, J.B. Hong, B. Melnik, O. Yamasaki, C. Dessinioti, Q. Ju, A. Liakou, S. Al-Khuzaei, A. Katsambas, J. Ring, C. Zouboulis (2010) Acne-associated syndromes: models for better understanding of acne pathogenesis. *J Eur Acad Dermatol Venereol*.
- Cohen, M.M., Jr. (1975) An etiologic and nosologic overview of craniosynostosis syndromes. *Birth Defects Orig Artic Ser* 11(2): 137-189.
- Cohen, M.M., Jr., S. Kreiborg (1990) The central nervous system in the Apert syndrome. *American journal of medical genetics* 35(1): 36-45.
- Cohen, M.M., Jr., S. Kreiborg (1991a) Agenesis of the corpus callosum. Its associated anomalies and syndromes with special reference to the Apert syndrome. *Neurosurgery clinics of North America* 2(3): 565-568.
- Cohen, M.M., Jr., S. Kreiborg (1991b) Genetic and family study of the Apert syndrome. *J Craniofac Genet Dev Biol* 11(1): 7-17.
- Cohen, M.M., Jr., S. Kreiborg (1993a) Growth pattern in the Apert syndrome. *American journal of medical genetics* 47(5): 617-623.
- Cohen, M.M., Jr., S. Kreiborg (1993b) Skeletal abnormalities in the Apert syndrome. *American journal of medical genetics* 47(5): 624-632.
- Cohen, M.M., Jr., S. Kreiborg (1993c) An updated pediatric perspective on the Apert syndrome. *Am J Dis Child* 147(9): 989-993.
- Cohen, M.M., Jr., S. Kreiborg (1993d) Visceral anomalies in the Apert syndrome. *American journal of medical genetics* 45(6): 758-760.
- Cohen, M.M., Jr., S. Kreiborg (1994) Cranial size and configuration in the Apert syndrome. *Journal of craniofacial genetics and developmental biology* 14(3): 153-162.
- Cohen, M.M., Jr., S. Kreiborg, E.J. Lammer, J.F. Cordero, P. Mastroiacovo, J.D. Erickson, P. Roeper, M.L. Martinez-Frias (1992) Birth prevalence study of the Apert syndrome. *American journal of medical genetics* 42(5): 655-659.
- Cohen, M.M.J.M., R. E (2000) *Craniosynostosis: diagnosis, evaluation and management*. USA, Oxford University Press.
- Colnot, C. (2009) Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. *J Bone Miner Res* 24(2): 274-282.
- Colvin, J.S., R.P. Green, J. Schmahl, B. Capel, D.M. Ornitz (2001a) Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* 104(6): 875-889.
- Colvin, J.S., A.C. White, S.J. Pratt, D.M. Ornitz (2001b) Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. *Development (Cambridge, England)* 128(11): 2095-2106.

- Cunningham, M.L., M.L. Seto, C. Ratisoontorn, C.L. Heike, A.V. Hing (2007) Syndromic craniosynostosis: from history to hydrogen bonds. *Orthod Craniofac Res* 10(2): 67-81.
- De Bari, C., F. Dell'Accio, J. Vanlauwe, J. Eyckmans, I.M. Khan, C.W. Archer, E.A. Jones, D. McGonagle, T.A. Mitsiadis, C. Pitzalis, F.P. Luyten (2006) Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. *Arthritis and rheumatism* 54(4): 1209-1221.
- de Leon, G.A., G. de Leon, W.D. Grover, N. Zaeri, P.D. Alburger (1987) Agenesis of the corpus callosum and limbic malformation in Apert syndrome (type I acrocephalosyndactyly). *Archives of neurology* 44(9): 979-982.
- de Mendonca Costa, A., D.F. Bueno, M.T. Martins, I. Kerkis, A. Kerkis, R.D. Fanganiello, H. Cerruti, N. Alonso, M.R. Passos-Bueno (2008) Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells. *The Journal of craniofacial surgery* 19(1): 204-210.
- Dono, R., G. Texido, R. Dussel, H. Ehmke, R. Zeller (1998) Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *The EMBO journal* 17(15): 4213-4225.
- Drafahl, K.A., C.W. McAndrew, A.N. Meyer, M. Haas, D.J. Donoghue (2010) The receptor tyrosine kinase FGFR4 negatively regulates NF-kappaB signaling. *PloS one* 5(12): e14412.
- Ducy, P., M. Starbuck, M. Priemel, J. Shen, G. Pinero, V. Geoffroy, M. Amling, G. Karsenty (1999) A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes & development* 13(8): 1025-1036.
- Eckenstein, F., W.R. Woodward, R. Nishi (1991) Differential localization and possible functions of aFGF and bFGF in the central and peripheral nervous systems. *Annals of the New York Academy of Sciences* 638: 348-360.
- Eckenstein, F.P. (1994) Fibroblast growth factors in the nervous system. *Journal of neurobiology* 25(11): 1467-1480.
- Emoto, N., A.M. Gonzalez, P.A. Walicke, E. Wada, D.M. Simmons, S. Shimasaki, A. Baird (1989) Basic fibroblast growth factor (FGF) in the central nervous system: identification of specific loci of basic FGF expression in the rat brain. *Growth factors (Chur, Switzerland)* 2(1): 21-29.
- Eswarakumar, V.P., E. Monsonogo-Ornan, M. Pines, I. Antonopoulou, G.M. Morriss-Kay, P. Lonai (2002) The *Il1c* alternative of *Fgfr2* is a positive regulator of bone formation. *Development (Cambridge, England)* 129(16): 3783-3793.
- Fanganiello, R.D., A.L. Sertie, E.M. Reis, E. Yeh, N.A. Oliveira, D.F. Bueno, I. Kerkis, N. Alonso, S. Cavaleiro, H. Matsushita, R. Freitas, S. Verjovski-Almeida, M.R. Passos-Bueno (2007) Apert p.Ser252Trp mutation in FGFR2 alters osteogenic potential and gene expression of cranial periosteal cells. *Molecular medicine (Cambridge, Mass)* 13(7-8): 422-442.
- Feldman, B., W. Poueymirou, V.E. Papaioannou, T.M. DeChiara, M. Goldfarb (1995) Requirement of FGF-4 for postimplantation mouse development. *Science (New York, NY)* 267(5195): 246-249.
- Floss, T., H.H. Arnold, T. Braun (1997) A role for FGF-6 in skeletal muscle regeneration. *Genes & development* 11(16): 2040-2051.
- Foster, K.A., D.M. Frim, M. McKinnon (2008) Recurrence of synostosis following surgical repair of craniosynostosis. *Plastic and reconstructive surgery* 121(3): 70e-76e.
- Fragale, A., M. Tartaglia, S. Bernardini, A.M. Di Stasi, C. Di Rocco, F. Velardi, A. Teti, P.A. Battaglia, S. Migliaccio (1999) Decreased proliferation and altered differentiation in osteoblasts from genetically and clinically distinct craniosynostotic disorders. *The American journal of pathology* 154(5): 1465-1477.
- Franceschi, R.T., G. Xiao (2003) Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. *Journal of cellular biochemistry* 88(3): 446-454.
- Franz-Odenaal, T.A., B.K. Hall, P.E. Witten (2006) Buried alive: how osteoblasts become osteocytes. *Developmental dynamics : an official publication of the American Association of Anatomists* 235(1): 176-190.
- Freshney, R.I. (2005) *Culture of Animal Cells: A Manual of Basic Technique*, Wiley-Liss.
- Furdui, C.M., E.D. Lew, J. Schlessinger, K.S. Anderson (2006) Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction. *Molecular cell* 21(5): 711-717.
- Gallea, S., F. Lallemand, A. Atfi, G. Rawadi, V. Ramez, S. Spinella-Jaegle, S. Kawai, C. Faucheu, L. Huet, R. Baron, S. Roman-Roman (2001) Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. *Bone* 28(5): 491-498.

- Gazzerro, E., V. Gangji, E. Canalis (1998) Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. *The Journal of clinical investigation* 102(12): 2106-2114.
- Gilbert, S.F. (2006) *Developmental Biology*, Sinauer Associates, Inc. 7th edition.
- Givol, D., A. Yayon (1992) Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *Faseb J* 6(15): 3362-3369.
- Glaser, R.L., K.W. Broman, R.L. Schulman, B. Eskenazi, A.J. Wyrobek, E.W. Jabs (2003) The paternal-age effect in Apert syndrome is due, in part, to the increased frequency of mutations in sperm. *American journal of human genetics* 73(4): 939-947.
- Goetz, R., A. Beenken, O.A. Ibrahimi, J. Kalinina, S.K. Olsen, A.V. Eliseenkova, C. Xu, T.A. Neubert, F. Zhang, R.J. Linhardt, X. Yu, K.E. White, T. Inagaki, S.A. Kliewer, M. Yamamoto, H. Kurosu, Y. Ogawa, M. Kuro-o, B. Lanske, M.S. Razzaque, M. Mohammadi (2007) Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Molecular and cellular biology* 27(9): 3417-3428.
- Goetz, S.C., K.V. Anderson (2010) The primary cilium: a signalling centre during vertebrate development. *Nature reviews* 11(5): 331-344.
- Gosain, A., L.A. DiPietro (2004) Aging and wound healing. *World journal of surgery* 28(3): 321-326.
- Gregory, C.A., W.G. Gunn, A. Peister, D.J. Prockop (2004) An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Analytical biochemistry* 329(1): 77-84.
- Guenou, H., K. Kaabeche, S.L. Mee, P.J. Marie (2005) A role for fibroblast growth factor receptor-2 in the altered osteoblast phenotype induced by Twist haploinsufficiency in the Saethre-Chotzen syndrome. *Human molecular genetics* 14(11): 1429-1439.
- Guicheux, J., J. Lemonnier, C. Ghayor, A. Suzuki, G. Palmer, J. Caverzasio (2003) Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *J Bone Miner Res* 18(11): 2060-2068.
- Guo, L., L. Degenstein, E. Fuchs (1996) Keratinocyte growth factor is required for hair development but not for wound healing. *Genes & development* 10(2): 165-175.
- Hacker, U., K. Nybakken, N. Perrimon (2005) Heparan sulphate proteoglycans: the sweet side of development. *Nature reviews Molecular cell biology* 6(7): 530-541.
- Hafler, D.A., A. Compston, S. Sawcer, E.S. Lander, M.J. Daly, P.L. De Jager, P.I. de Bakker, S.B. Gabriel, D.B. Mirel, A.J. Iverson, M.A. Pericak-Vance, S.G. Gregory, J.D. Rioux, J.L. McCauley, J.L. Haines, L.F. Barcellos, B. Cree, J.R. Oksenberg, S.L. Hauser (2007) Risk alleles for multiple sclerosis identified by a genomewide study. *The New England journal of medicine* 357(9): 851-862.
- Hajihosseini, M.K. (2008) Fibroblast growth factor signaling in cranial suture development and pathogenesis. *Frontiers of oral biology* 12: 160-177.
- Hajihosseini, M.K., R. Duarte, J. Pegrum, A. Donjacour, E. Lana-Elola, D.P. Rice, J. Sharpe, C. Dickson (2009) Evidence that Fgf10 contributes to the skeletal and visceral defects of an Apert syndrome mouse model. *Developmental dynamics : an official publication of the American Association of Anatomists* 238(2): 376-385.
- Hajihosseini, M.K., J.K. Heath (2002) Expression patterns of fibroblast growth factors-18 and -20 in mouse embryos is suggestive of novel roles in calvarial and limb development. *Mechanisms of development* 113(1): 79-83.
- Hajihosseini, M.K., S. Wilson, L. De Moerloose, C. Dickson (2001) A splicing switch and gain-of-function mutation in FgfR2-IIIc hemizygotes causes Apert/Pfeiffer-syndrome-like phenotypes. *Proceedings of the National Academy of Sciences of the United States of America* 98(7): 3855-3860.
- Hall, M., F. Gofflot, S. Iseki, G.M. Morriss-Kay (2001) Effects of the curly tail genotype on neuroepithelial integrity and cell proliferation during late stages of primary neurulation. *Journal of anatomy* 199(Pt 6): 645-655.
- Hart, A.W., N. Baeza, A. Apelqvist, H. Edlund (2000) Attenuation of FGF signalling in mouse beta-cells leads to diabetes. *Nature* 408(6814): 864-868.
- Hauschka, P.V., F.H. Wians, Jr. (1989) Osteocalcin-hydroxyapatite interaction in the extracellular organic matrix of bone. *The Anatomical record* 224(2): 180-188.
- Hebert, J.M., T. Rosenquist, J. Gotz, G.R. Martin (1994) FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* 78(6): 1017-1025.
- Holmes, G., G. Rothschild, U.B. Roy, C.X. Deng, A. Mansukhani, C. Basilico (2009) Early onset of craniosynostosis in an Apert mouse model reveals critical features of this pathology. *Developmental biology* 328(2): 273-284.

- Hong, F., R. Breitling, C.W. McEntee, B.S. Wittner, J.L. Nemhauser, J. Chory (2006) RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics* (Oxford, England) 22(22): 2825-2827.
- Hopper, R.A., J.R. Zhang, V.L. Fourasier, I. Morova-Protzner, K.F. Protzner, C.Y. Pang, C.R. Forrest (2001) Effect of isolation of periosteum and dura on the healing of rabbit calvarial inlay bone grafts. *Plastic and reconstructive surgery* 107(2): 454-462.
- Hunter, T. (2007) The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Molecular cell* 28(5): 730-738.
- Hurley, J.H., S. Lee, G. Prag (2006) Ubiquitin-binding domains. *The Biochemical journal* 399(3): 361-372.
- Ibrahimi, O.A., A.V. Eliseenkova, A.N. Plotnikov, K. Yu, D.M. Ornitz, M. Mohammadi (2001) Structural basis for fibroblast growth factor receptor 2 activation in Apert syndrome. *Proceedings of the National Academy of Sciences of the United States of America* 98(13): 7182-7187.
- Ibrahimi, O.A., B.K. Yeh, A.V. Eliseenkova, F. Zhang, S.K. Olsen, M. Igarashi, S.A. Aaronson, R.J. Linhardt, M. Mohammadi (2005) Analysis of mutations in fibroblast growth factor (FGF) and a pathogenic mutation in FGF receptor (FGFR) provides direct evidence for the symmetric two-end model for FGFR dimerization. *Molecular and cellular biology* 25(2): 671-684.
- Ibrahimi, O.A., F. Zhang, A.V. Eliseenkova, N. Itoh, R.J. Linhardt, M. Mohammadi (2004) Biochemical analysis of pathogenic ligand-dependent FGFR2 mutations suggests distinct pathophysiological mechanisms for craniofacial and limb abnormalities. *Human molecular genetics* 13(19): 2313-2324.
- Ito, Y., A. Sanyal, J.S. Fitzsimmons, M.A. Mello, S.W. O'Driscoll (2001) Histomorphological and proliferative characterization of developing periosteal neochondrocytes in vitro. *J Orthop Res* 19(3): 405-413.
- Itoh, N., D.M. Ornitz (2004) Evolution of the Fgf and Fgfr gene families. *Trends Genet* 20(11): 563-569.
- Itoh, N., D.M. Ornitz (2008) Functional evolutionary history of the mouse Fgf gene family. *Developmental dynamics : an official publication of the American Association of Anatomists* 237(1): 18-27.
- Jaslow, C.R. (1990) Mechanical properties of cranial sutures. *J Biomech* 23(4): 313-321.
- Jiang, X., S. Iseki, R.E. Maxson, H.M. Sucov, G.M. Morriss-Kay (2002) Tissue origins and interactions in the mammalian skull vault. *Developmental biology* 241(1): 106-116.
- Johnson, D. (2003) A comprehensive screen of genes implicated in craniosynostosis. *Annals of the Royal College of Surgeons of England* 85(6): 371-377.
- Johnson, D., A.O. Wilkie (2011) Craniosynostosis. *Eur J Hum Genet* 19(4): 369-376.
- Johnson, D.E., L.T. Williams (1993) Structural and functional diversity in the FGF receptor multigene family. *Advances in cancer research* 60: 1-41.
- Joiakim, A., P.A. Mathieu, C. Palermo, T.A. Gasiewicz, J.J. Reiners, Jr. (2003) The Jun N-terminal kinase inhibitor SP600125 is a ligand and antagonist of the aryl hydrocarbon receptor. *Drug metabolism and disposition: the biological fate of chemicals* 31(11): 1279-1282.
- Kalluri, R., R.A. Weinberg (2009) The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation* 119(6): 1420-1428.
- Kasser, J., J. Upton (1991) The shoulder, elbow, and forearm in Apert syndrome. *Clin Plast Surg* 18(2): 381-389.
- Kim, H.J., J.H. Kim, S.C. Bae, J.Y. Choi, H.M. Ryoo (2003) The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. *The Journal of biological chemistry* 278(1): 319-326.
- Kirkin, V., I. Dikic (2007) Role of ubiquitin- and Ubl-binding proteins in cell signaling. *Curr Opin Cell Biol* 19(2): 199-205.
- Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R.T. Bronson, Y.H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki, T. Kishimoto (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89(5): 755-764.
- Kouhara, H., Y.R. Hadari, T. Spivak-Kroizman, J. Schilling, D. Bar-Sagi, I. Lax, J. Schlessinger (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* 89(5): 693-702.
- Ku, D.H., C.D. Chang, J. Koniecki, L.A. Cannizzaro, L. Boghosian-Sell, H. Alder, R. Baserga (1991) A new growth-regulated complementary DNA with the sequence of a putative trans-activating factor. *Cell Growth Differ* 2(4): 179-186.
- Kuratani, S., I. Matsuo, S. Aizawa (1997) Developmental patterning and evolution of the mammalian viscerocranium: genetic insights into comparative morphology. *Developmental dynamics : an official publication of the American Association of Anatomists* 209(2): 139-155.

- Kurosu, H., M. Choi, Y. Ogawa, A.S. Dickson, R. Goetz, A.V. Eliseenkova, M. Mohammadi, K.P. Rosenblatt, S.A. Kliewer, M. Kuro-o (2007) Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *The Journal of biological chemistry* 282(37): 26687-26695.
- Ladbury, J.E., S. Arold (2000) Searching for specificity in SH domains. *Chem Biol* 7(1): R3-8.
- Ladher, R.K., K.U. Anakwe, A.L. Gurney, G.C. Schoenwolf, P.H. Francis-West (2000) Identification of synergistic signals initiating inner ear development. *Science (New York, NY)* 290(5498): 1965-1967.
- Lajeunie, E., R. Cameron, V. El Ghouzzi, N. de Parseval, P. Journeau, M. Gonzales, A.L. Delezoide, J. Bonaventure, M. Le Merrer, D. Renier (1999) Clinical variability in patients with Apert's syndrome. *J Neurosurg* 90(3): 443-447.
- Lajeunie, E., M. Le Merrer, C. Bonaiti-Pellie, D. Marchac, D. Renier (1995) Genetic study of nonsyndromic coronal craniosynostosis. *American journal of medical genetics* 55(4): 500-504.
- Langille, R.M. (1994) Chondrogenic differentiation in cultures of embryonic rat mesenchyme. *Microsc Res Tech* 28(6): 455-469.
- Lawson, K.A., R.A. Pedersen (1992) Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. *Ciba Found Symp* 165: 3-21; discussion 21-26.
- Lee, D.S., K.C. Chung (2010) Eugene Apert and his contributions to plastic surgery. *Ann Plast Surg* 64(3): 362-365.
- Legeai-Mallet, L., C. Benoist-Lassel, A.L. Delezoide, A. Munnich, J. Bonaventure (1998) Fibroblast growth factor receptor 3 mutations promote apoptosis but do not alter chondrocyte proliferation in thanatophoric dysplasia. *The Journal of biological chemistry* 273(21): 13007-13014.
- Lemmon, M.A., J. Schlessinger (2010) Cell signaling by receptor tyrosine kinases. *Cell* 141(7): 1117-1134.
- Lemonnier, J., E. Hay, P. Delannoy, O. Fromigue, A. Lomri, D. Modrowski, P.J. Marie (2001a) Increased osteoblast apoptosis in apert craniosynostosis: role of protein kinase C and interleukin-1. *The American journal of pathology* 158(5): 1833-1842.
- Lemonnier, J., E. Hay, P. Delannoy, A. Lomri, D. Modrowski, J. Caverzasio, P.J. Marie (2001b) Role of N-cadherin and protein kinase C in osteoblast gene activation induced by the S252W fibroblast growth factor receptor 2 mutation in Apert craniosynostosis. *J Bone Miner Res* 16(5): 832-845.
- Lenton, K.A., R.P. Nacamuli, D.C. Wan, J.A. Helms, M.T. Longaker (2005) Cranial suture biology. *Current topics in developmental biology* 66: 287-328.
- Levine, J.P., J.P. Bradley, D.A. Roth, J.G. McCarthy, M.T. Longaker (1998) Studies in cranial suture biology: regional dura mater determines overlying suture biology. *Plastic and reconstructive surgery* 101(6): 1441-1447.
- Li, C., D.A. Scott, E. Hatch, X. Tian, S.L. Mansour (2007) Dusp6 (Mkp3) is a negative feedback regulator of FGF-stimulated ERK signaling during mouse development. *Development (Cambridge, England)* 134(1): 167-176.
- Li, H., S. Wetten, L. Li, P.L. St Jean, R. Upmanyu, L. Surh, D. Hosford, M.R. Barnes, J.D. Briley, M. Borrie, N. Coletta, R. Delisle, D. Dhalla, M.G. Ehm, H.H. Feldman, L. Fornazzari, S. Gauthier, N. Goodgame, D. Guzman, S. Hammond, P. Hollingworth, G.Y. Hsiung, J. Johnson, D.D. Kelly, R. Keren, A. Kertesz, K.S. King, S. Lovestone, I. Loy-English, P.M. Matthews, M.J. Owen, M. Plumpton, W. Pryse-Phillips, R.K. Prinjha, J.C. Richardson, A. Saunders, A.J. Slater, P.H. St George-Hyslop, S.W. Stinnett, J.E. Swartz, R.L. Taylor, J. Wherrett, J. Williams, D.P. Yarnall, R.A. Gibson, M.C. Irizarry, L.T. Middleton, A.D. Roses (2008) Candidate single-nucleotide polymorphisms from a genomewide association study of Alzheimer disease. *Archives of neurology* 65(1): 45-53.
- Lilli, C., C. Bellucci, T. Baroni, C. Aisa, P. Carinci, L. Scapoli, F. Carinci, F. Pezzetti, E. Lumare, G. Stabellini, M. Bodo (2007) FGF2 effects in periosteal fibroblasts bearing the FGFR2 receptor Pro253 Arg mutation. *Cytokine* 38(1): 22-31.
- Liu, H., Y. Liu, M. Viggewarapu, Z. Zheng, L. Titus, S.D. Boden (2010) Activation of c-Jun NH(2)-terminal kinase 1 increases cellular responsiveness to BMP-2 and decreases binding of inhibitory Smad6 to the type I BMP receptor. *J Bone Miner Res*.
- Liu, Z., J. Xu, J.S. Colvin, D.M. Ornitz (2002) Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes & development* 16(7): 859-869.
- Lomri, A., J. Lemonnier, P. Delannoy, P.J. Marie (2001) Increased expression of protein kinase Calpha, interleukin-1alpha, and RhoA guanosine 5'-triphosphatase in osteoblasts expressing the Ser252Trp fibroblast growth factor 2 receptor Apert mutation: identification by analysis of complementary DNA microarray. *J Bone Miner Res* 16(4): 705-712.

- Lomri, A., J. Lemonnier, M. Hott, N. de Parseval, E. Lajeunie, A. Munnich, D. Renier, P.J. Marie (1998) Increased calvaria cell differentiation and bone matrix formation induced by fibroblast growth factor receptor 2 mutations in Apert syndrome. *The Journal of clinical investigation* 101(6): 1310-1317.
- Lopez-Bergami, P., C. Huang, J.S. Goydos, D. Yip, M. Bar-Eli, M. Herlyn, K.S. Smalley, A. Mahale, A. Eroshkin, S. Aaronson, Z. Ronai (2007) Rewired ERK-JNK signaling pathways in melanoma. *Cancer cell* 11(5): 447-460.
- Mangasarian, K., Y. Li, A. Mansukhani, C. Basilico (1997) Mutation associated with Crouzon syndrome causes ligand-independent dimerization and activation of FGF receptor-2. *Journal of cellular physiology* 172(1): 117-125.
- Mansour, S.L., J.M. Goddard, M.R. Capecchi (1993) Mice homozygous for a targeted disruption of the proto-oncogene int-2 have developmental defects in the tail and inner ear. *Development* (Cambridge, England) 117(1): 13-28.
- Mansukhani, A., P. Bellosta, M. Sahni, C. Basilico (2000) Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. *The Journal of cell biology* 149(6): 1297-1308.
- Marshall, M.S. (1995) Ras target proteins in eukaryotic cells. *Faseb J* 9(13): 1311-1318.
- Matsuguchi, T., N. Chiba, K. Bandow, K. Kakimoto, A. Masuda, T. Ohnishi (2009) JNK activity is essential for Atf4 expression and late-stage osteoblast differentiation. *J Bone Miner Res* 24(3): 398-410.
- Matsunaga, S., M. Okigaki, M. Takeda, A. Matsui, S. Honsho, A. Katsume, E. Kishita, J. Che, T. Kurihara, Y. Adachi, A. Mansukhani, M. Kobara, S. Matoba, T. Tatsumi, H. Matsubara (2009) Endothelium-targeted overexpression of constitutively active FGF receptor induces cardioprotection in mice myocardial infarction. *Journal of molecular and cellular cardiology* 46(5): 663-673.
- McHugh, T., M. Wyers, E. King (2007) MRI characterization of the glenohumeral joint in Apert syndrome. *Pediatric radiology* 37(6): 596-599.
- Merrill, A.E., E.G. Bochukova, S.M. Brugger, M. Ishii, D.T. Pilz, S.A. Wall, K.M. Lyons, A.O. Wilkie, R.E. Maxson, Jr. (2006) Cell mixing at a neural crest-mesoderm boundary and deficient ephrin-Eph signaling in the pathogenesis of craniosynostosis. *Human molecular genetics* 15(8): 1319-1328.
- Meyers, E.N., M. Lewandoski, G.R. Martin (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nature genetics* 18(2): 136-141.
- Miki, T., D.P. Bottaro, T.P. Fleming, C.L. Smith, W.H. Burgess, A.M. Chan, S.A. Aaronson (1992) Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proceedings of the National Academy of Sciences of the United States of America* 89(1): 246-250.
- Miller, D.L., S. Ortega, O. Bashayan, R. Basch, C. Basilico (2000) Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Molecular and cellular biology* 20(6): 2260-2268.
- Min, H., D.M. Danilenko, S.A. Scully, B. Bolon, B.D. Ring, J.E. Tarpley, M. DeRose, W.S. Simonet (1998) Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to *Drosophila* branchless. *Genes & development* 12(20): 3156-3161.
- Miraoui, H., P.J. Marie (2010) Fibroblast growth factor receptor signaling crosstalk in skeletogenesis. *Sci Signal* 3(146): re9.
- Miraoui, H., K. Oudina, H. Petite, Y. Tanimoto, K. Moriyama, P.J. Marie (2009) Fibroblast growth factor receptor 2 promotes osteogenic differentiation in mesenchymal cells via ERK1/2 and protein kinase C signaling. *The Journal of biological chemistry* 284(8): 4897-4904.
- Miraoui, H., J. Ringe, T. Haupl, P.J. Marie (2010) Increased EFG- and PDGFalpha-receptor signaling by mutant FGF-receptor 2 contributes to osteoblast dysfunction in Apert craniosynostosis. *Human molecular genetics* 19(9): 1678-1689.
- Miura, T., C.A. Perlyn, M. Kinboshi, N. Ogihara, M. Kobayashi-Miura, G.M. Morriss-Kay, K. Shiota (2009) Mechanism of skull suture maintenance and interdigitation. *Journal of anatomy* 215(6): 642-655.
- Mohammadi, M., S.K. Olsen, O.A. Ibrahimi (2005) Structural basis for fibroblast growth factor receptor activation. *Cytokine & growth factor reviews* 16(2): 107-137.
- Moloney, D.M., S.F. Slaney, M. Oldridge, S.A. Wall, P. Sahlin, G. Stenman, A.O. Wilkie (1996) Exclusive paternal origin of new mutations in Apert syndrome. *Nature genetics* 13(1): 48-53.
- Montero, A., Y. Okada, M. Tomita, M. Ito, H. Tsurukami, T. Nakamura, T. Doetschman, J.D. Coffin, M.M. Hurley (2000) Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *The Journal of clinical investigation* 105(8): 1085-1093.

- Morriss-Kay, G.M., A.O. Wilkie (2005) Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. *Journal of anatomy* 207(5): 637-653.
- Munroe, P.B., R.O. Olgunturk, J.P. Fryns, L. Van Maldergem, F. Ziereisen, B. Yuksel, R.M. Gardiner, E. Chung (1999) Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. *Nature genetics* 21(1): 142-144.
- Murnaghan, L.M., C.H. Thurgur, B.B. Forster, B.J. Sawatzky, R. Hawkins, S.J. Tredwell (2007) A clinoradiologic study of the shoulder in Apert syndrome. *Journal of pediatric orthopedics* 27(7): 838-843.
- Nair, R.P., P.E. Stuart, I. Nistor, R. Hiremagalore, N.V. Chia, S. Jenisch, M. Weichenthal, G.R. Abecasis, H.W. Lim, E. Christophers, J.J. Voorhees, J.T. Elder (2006) Sequence and haplotype analysis supports HLA-C as the psoriasis susceptibility 1 gene. *American journal of human genetics* 78(5): 827-851.
- Naski, M.C., D.M. Ornitz (1998) FGF signaling in skeletal development. *Front Biosci* 3: d781-794.
- Neugebauer, J.M., J.D. Amack, A.G. Peterson, B.W. Bisgrove, H.J. Yost (2009) FGF signalling during embryo development regulates cilia length in diverse epithelia. *Nature* 458(7238): 651-654.
- Nickel, W. (2005) Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells. *Traffic* 6(8): 607-614.
- Ninomiya, J.T., R.P. Tracy, J.D. Calore, M.A. Gendreau, R.J. Kelm, K.G. Mann (1990) Heterogeneity of human bone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 5(9): 933-938.
- Nomura, S., H. Yoshitomi, S. Takano, T. Shida, S. Kobayashi, M. Ohtsuka, F. Kimura, H. Shimizu, H. Yoshidome, A. Kato, M. Miyazaki (2008) FGF10/FGFR2 signal induces cell migration and invasion in pancreatic cancer. *British journal of cancer* 99(2): 305-313.
- Ohbayashi, N., M. Shibayama, Y. Kurotaki, M. Imanishi, T. Fujimori, N. Itoh, S. Takada (2002) FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes & development* 16(7): 870-879.
- Ohkido, I., K. Yokoyama, S. Kagami, T. Hosoya (2010) The hypothesis that bone turnover influences FGF23 secretion. *Kidney international* 77(8): 743; author reply 743-744.
- Oldridge, M., P.W. Lunt, E.H. Zackai, D.M. McDonald-McGinn, M. Muenke, D.M. Moloney, S.R. Twigg, J.K. Heath, T.D. Howard, G. Hoganson, D.M. Gagnon, E.W. Jabs, A.O. Wilkie (1997) Genotype-phenotype correlation for nucleotide substitutions in the IgII-IgIII linker of FGFR2. *Human molecular genetics* 6(1): 137-143.
- Oldridge, M., E.H. Zackai, D.M. McDonald-McGinn, S. Iseki, G.M. Morriss-Kay, S.R. Twigg, D. Johnson, S.A. Wall, W. Jiang, C. Theda, E.W. Jabs, A.O. Wilkie (1999) De novo alu-element insertions in FGFR2 identify a distinct pathological basis for Apert syndrome. *American journal of human genetics* 64(2): 446-461.
- Opperman, L.A. (2000) Cranial sutures as intramembranous bone growth sites. *Developmental dynamics : an official publication of the American Association of Anatomists* 219(4): 472-485.
- Opperman, L.A., T.M. Sweeney, J. Redmon, J.A. Persing, R.C. Ogle (1993) Tissue interactions with underlying dura mater inhibit osseous obliteration of developing cranial sutures. *Developmental dynamics : an official publication of the American Association of Anatomists* 198(4): 312-322.
- Ornitz, D.M. (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays* 22(2): 108-112.
- Ornitz, D.M., J. Xu, J.S. Colvin, D.G. McEwen, C.A. MacArthur, F. Coulier, G. Gao, M. Goldfarb (1996) Receptor specificity of the fibroblast growth factor family. *The Journal of biological chemistry* 271(25): 15292-15297.
- Orr-Urtreger, A., M.T. Bedford, T. Burakova, E. Arman, Y. Zimmer, A. Yayon, D. Givol, P. Lonai (1993) Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Developmental biology* 158(2): 475-486.
- Orwoll, E. (2006) Genetic aspects of osteoporosis. *Annales d'endocrinologie* 67(2): 113.
- Ozerdem, O.R., R. Anlatıcı, T. Bahar, F. Kayaselcuk, O. Barutcu, I. Tuncer, O. Sen (2003) Roles of periosteum, dura, and adjacent bone on healing of cranial osteonecrosis. *The Journal of craniofacial surgery* 14(3): 371-379; discussion 380-372.
- Park, I., J. Chung, C.T. Walsh, Y. Yun, J.L. Strominger, J. Shin (1995a) Phosphotyrosine-independent binding of a 62-kDa protein to the src homology 2 (SH2) domain of p56lck and its regulation by phosphorylation of Ser-59 in the lck unique N-terminal region. *Proceedings of the National Academy of Sciences of the United States of America* 92(26): 12338-12342.

- Park, W.J., C. Theda, N.E. Maestri, G.A. Meyers, J.S. Fryburg, C. Dufresne, M.M. Cohen, Jr., E.W. Jabs (1995b) Analysis of phenotypic features and FGFR2 mutations in Apert syndrome. *American journal of human genetics* 57(2): 321-328.
- Passos-Bueno, M.R., A. Richieri-Costa, A.L. Sertie, A. Kneppers (1998) Presence of the Apert canonical S252W FGFR2 mutation in a patient without severe syndactyly. *J Med Genet* 35(8): 677-679.
- Passos-Bueno, M.R., A.E. Serti Eacute, F.S. Jehee, R. Fanganiello, E. Yeh (2008) Genetics of craniosynostosis: genes, syndromes, mutations and genotype-phenotype correlations. *Frontiers of oral biology* 12: 107-143.
- Passos-Bueno, M.R., A.L. Sertie, M. Zatz, A. Richieri-Costa (1997) Pfeiffer mutation in an Apert patient: how wide is the spectrum of variability due to mutations in the FGFR2 gene? *American journal of medical genetics* 71(2): 243-245.
- Patterson, K.I., T. Brummer, P.M. O'Brien, R.J. Daly (2009) Dual-specificity phosphatases: critical regulators with diverse cellular targets. *The Biochemical journal* 418(3): 475-489.
- Pawson, T. (2004) Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell* 116(2): 191-203.
- Persson, M. (1995) The role of sutures in normal and abnormal craniofacial growth. *Acta Odontol Scand* 53(3): 152-161.
- Posnick, J.C., M.M. al-Qattan, S.M. Moffat, D. Armstrong (1995) Cranio-orbito-zygomatic measurements from standard CT scans in unoperated Treacher Collins syndrome patients: comparison with normal controls. *Cleft Palate Craniofac J* 32(1): 20-24.
- Putz, R., Pabst, R. (2001) *Sobotta Atlas of Human Anatomy. Volume 1: Head, Neck, Upper limb.* Lippincott Williams & Wilkins; Thirteenth edition.
- Quarto, N., D.C. Wan, M.T. Longaker (2008) Molecular mechanisms of FGF-2 inhibitory activity in the osteogenic context of mouse adipose-derived stem cells (mASCs). *Bone* 42(6): 1040-1052.
- Quintero-Rivera, F., C.D. Robson, R.E. Reiss, D. Levine, C.B. Benson, J.B. Mulliken, V.E. Kimonis (2006) Intracranial anomalies detected by imaging studies in 30 patients with Apert syndrome. *Am J Med Genet A* 140(12): 1337-1338.
- Rasband, W.S. (1997-2009) *ImageJ.* Bethesda, Maryland, USA, U. S. National Institutes of Health.
- Ratisoontorn, C., G.F. Fan, K. McEntee, H.D. Nah (2003) Activating (P253R, C278F) and dominant negative mutations of FGFR2: differential effects on calvarial bone cell proliferation, differentiation, and mineralization. *Connect Tissue Res* 44 Suppl 1: 292-297.
- Raybaud, C., C. Di Rocco (2007) Brain malformation in syndromic craniosynostoses, a primary disorder of white matter: a review. *Childs Nerv Syst* 23(12): 1379-1388.
- Razzaque, M.S. (2009) FGF23-mediated regulation of systemic phosphate homeostasis: is Klotho an essential player? *American journal of physiology* 296(3): F470-476.
- Renier, D., E. Arnaud, G. Cinalli, D. Marchac, L. Brunet, G. Sebag, C. Sainte-Rose, M. Zerah (1996) [Mental prognosis of Apert syndrome]. *Archives de pediatrie : organe officiel de la Societe francaise de pediatrie* 3(8): 752-760.
- Renier, D., E. Lajeunie, E. Arnaud, D. Marchac (2000) Management of craniosynostoses. *Childs Nerv Syst* 16(10-11): 645-658.
- Rice, D.P. (2008) Developmental anatomy of craniofacial sutures. *Frontiers of oral biology* 12: 1-21.
- Rice, D.P., R. Rice (2008) Locate, condense, differentiate, grow and confront: developmental mechanisms controlling intramembranous bone and suture formation and function. *Frontiers of oral biology* 12: 22-40.
- Romberg, R.W., P.G. Werness, B.L. Riggs, K.G. Mann (1986) Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. *Biochemistry* 25(5): 1176-1180.
- Roth, D.A., J.P. Bradley, J.P. Levine, H.F. McMullen, J.G. McCarthy, M.T. Longaker (1996) Studies in cranial suture biology: part II. Role of the dura in cranial suture fusion. *Plastic and reconstructive surgery* 97(4): 693-699.
- Saeys, Y., I. Inza, P. Larranaga (2007) A review of feature selection techniques in bioinformatics. *Bioinformatics (Oxford, England)* 23(19): 2507-2517.
- Sahni, M., D.C. Ambrosetti, A. Mansukhani, R. Gertner, D. Levy, C. Basilico (1999) FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes & development* 13(11): 1361-1366.
- Sauka-Spengler, T., M. Bronner-Fraser (2008) A gene regulatory network orchestrates neural crest formation. *Nature reviews Molecular cell biology* 9(7): 557-568.
- Scadden, D.T. (2006) The stem-cell niche as an entity of action. *Nature* 441(7097): 1075-1079.

- Schlessinger, J., M.A. Lemmon (2003) SH2 and PTB domains in tyrosine kinase signaling. *Sci STKE* 2003(191): RE12.
- Schlessinger, J., A.N. Plotnikov, O.A. Ibrahimi, A.V. Eliseenkova, B.K. Yeh, A. Yayon, R.J. Linhardt, M. Mohammadi (2000) Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Molecular cell* 6(3): 743-750.
- Seet, B.T., I. Dikic, M.M. Zhou, T. Pawson (2006) Reading protein modifications with interaction domains. *Nature reviews Molecular cell biology* 7(7): 473-483.
- Sekine, K., H. Ohuchi, M. Fujiwara, M. Yamasaki, T. Yoshizawa, T. Sato, N. Yagishita, D. Matsui, Y. Koga, N. Itoh, S. Kato (1999) Fgf10 is essential for limb and lung formation. *Nature genetics* 21(1): 138-141.
- Shimada, T., M. Kakitani, Y. Yamazaki, H. Hasegawa, Y. Takeuchi, T. Fujita, S. Fukumoto, K. Tomizuka, T. Yamashita (2004) Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *The Journal of clinical investigation* 113(4): 561-568.
- Shirakihara, T., K. Horiguchi, K. Miyazawa, S. Ehata, T. Shibata, I. Morita, K. Miyazono, M. Saitoh (2011) TGF-beta regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *The EMBO journal* 30(4): 783-795.
- Shukla, V., X. Coumoul, R.H. Wang, H.S. Kim, C.X. Deng (2007) RNA interference and inhibition of MEK-ERK signaling prevent abnormal skeletal phenotypes in a mouse model of craniosynostosis. *Nature genetics* 39(9): 1145-1150.
- Slaney, S.F., M. Oldridge, J.A. Hurst, G.M. Moriss-Kay, C.M. Hall, M.D. Poole, A.O. Wilkie (1996) Differential effects of FGFR2 mutations on syndactyly and cleft palate in Apert syndrome. *American journal of human genetics* 58(5): 923-932.
- Slater, B.J., K.A. Lenton, M.D. Kwan, D.M. Gupta, D.C. Wan, M.T. Longaker (2008) Cranial sutures: a brief review. *Plastic and reconstructive surgery* 121(4): 170e-178e.
- Spivak-Kroizman, T., M.A. Lemmon, I. Dikic, J.E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, I. Lax (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* 79(6): 1015-1024.
- Squier, C.A., S. Ghoneim, C.R. Kremenak (1990) Ultrastructure of the periosteum from membrane bone. *Journal of anatomy* 171: 233-239.
- Strutz, F., H. Okada, C.W. Lo, T. Danoff, R.L. Carone, J.E. Tomaszewski, E.G. Neilson (1995) Identification and characterization of a fibroblast marker: FSP1. *The Journal of cell biology* 130(2): 393-405.
- Strutz, F., M. Zeisberg, F.N. Ziyadeh, C.Q. Yang, R. Kalluri, G.A. Muller, E.G. Neilson (2002) Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation. *Kidney international* 61(5): 1714-1728.
- Su, N., Q. Sun, C. Li, X. Lu, H. Qi, S. Chen, J. Yang, X. Du, L. Zhao, Q. He, M. Jin, Y. Shen, D. Chen, L. Chen (2010) Gain-of-function mutation in FGFR3 in mice leads to decreased bone mass by affecting both osteoblastogenesis and osteoclastogenesis. *Human molecular genetics* 19(7): 1199-1210.
- Sun, X., E.N. Meyers, M. Lewandoski, G.R. Martin (1999) Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes & development* 13(14): 1834-1846.
- Tanimoto, Y., M. Yokozeki, K. Hiura, K. Matsumoto, H. Nakanishi, T. Matsumoto, P.J. Marie, K. Moriyama (2004) A soluble form of fibroblast growth factor receptor 2 (FGFR2) with S252W mutation acts as an efficient inhibitor for the enhanced osteoblastic differentiation caused by FGFR2 activation in Apert syndrome. *The Journal of biological chemistry* 279(44): 45926-45934.
- Teraoka, Y., T.K. Naruse, A. Oka, Y. Matsuzawa, T. Shiina, M. Iizuka, K. Iwashita, A. Ozawa, H. Inoko (2000) Genetic polymorphisms in the cell growth regulated gene, SC1 telomeric of the HLA-C gene and lack of association of psoriasis vulgaris. *Tissue antigens* 55(3): 206-211.
- Terauchi, A., E.M. Johnson-Venkatesh, A.B. Toth, D. Javed, M.A. Sutton, H. Umemori (2010) Distinct FGFs promote differentiation of excitatory and inhibitory synapses. *Nature* 465(7299): 783-787.
- Thiery, J.P., J.P. Sleeman (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nature reviews Molecular cell biology* 7(2): 131-142.
- Thisse, B., C. Thisse (2005) Functions and regulations of fibroblast growth factor signaling during embryonic development. *Developmental biology* 287(2): 390-402.
- Tolarova, M.M., J.A. Harris, D.E. Ordway, K. Vargervik (1997) Birth prevalence, mutation rate, sex ratio, parents' age, and ethnicity in Apert syndrome. *American journal of medical genetics* 72(4): 394-398.
- Trainor, P., M.A. Nieto (2003) Jawsfest: new perspectives on neural crest lineages and morphogenesis. *Development (Cambridge, England)* 130(21): 5059-5063.
- Tsai, F.J., W.L. Hwu, S.P. Lin, J.G. Chang, T.R. Wang, C.H. Tsai (1998) Two common mutations 934C to G and

- 937C to G of fibroblast growth factor receptor 2 (FGFR2) gene in Chinese patients with Apert syndrome. *Human mutation Suppl 1*: 518-19.
- Tusher, V.G., R. Tibshirani, G. Chu (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America* 98(9): 5116-5121.
- Umemori, H., M.W. Linhoff, D.M. Ornitz, J.R. Sanes (2004) FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell* 118(2): 257-270.
- Usui, H., M. Shibayama, N. Ohbayashi, M. Konishi, S. Takada, N. Itoh (2004) Fgf18 is required for embryonic lung alveolar development. *Biochem Biophys Res Commun* 322(3): 887-892.
- Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7): RESEARCH0034.
- Verpy, E., M. Leibovici, N. Michalski, R.J. Goodyear, C. Houdon, D. Weil, G.P. Richardson, C. Petit (2010) Stereocilin connects outer hair cell stereocilia to one another and to the tectorial membrane. *The Journal of comparative neurology* 519(2): 194-210.
- Verpy, E., S. Masmoudi, I. Zwaenepoel, M. Leibovici, T.P. Hutchin, I. Del Castillo, S. Nouaille, S. Blanchard, S. Laine, J.L. Popot, F. Moreno, R.F. Mueller, C. Petit (2001) Mutations in a new gene encoding a protein of the hair bundle cause non-syndromic deafness at the DFNB16 locus. *Nature genetics* 29(3): 345-349.
- Verpy, E., D. Weil, M. Leibovici, R.J. Goodyear, G. Hamard, C. Houdon, G.M. Lefevre, J.P. Hardelin, G.P. Richardson, P. Avan, C. Petit (2008) Stereocilin-deficient mice reveal the origin of cochlear waveform distortions. *Nature* 456(7219): 255-258.
- Vincentz, J.W., J.R. McWhirter, C. Murre, A. Baldini, Y. Furuta (2005) Fgf15 is required for proper morphogenesis of the mouse cardiac outflow tract. *Genesis* 41(4): 192-201.
- von Gernet, S., A. Golla, Y. Ehrenfels, S. Schuffenhauer, J.D. Fairley (2000) Genotype-phenotype analysis in Apert syndrome suggests opposite effects of the two recurrent mutations on syndactyly and outcome of craniofacial surgery. *Clin Genet* 57(2): 137-139.
- Wang, F., M. Kan, G. Yan, J. Xu, W.L. McKeenan (1995) Alternately spliced NH2-terminal immunoglobulin-like Loop I in the ectodomain of the fibroblast growth factor (FGF) receptor 1 lowers affinity for both heparin and FGF-1. *The Journal of biological chemistry* 270(17): 10231-10235.
- Wang, Y., R. Xiao, F. Yang, B.O. Karim, A.J. Iacovelli, J. Cai, C.P. Lerner, J.T. Richtsmeier, J.M. Leszl, C.A. Hill, K. Yu, D.M. Ornitz, J. Elisseeff, D.L. Huso, E.W. Jabs (2005) Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/-S252W) mouse. *Development (Cambridge, England)* 132(15): 3537-3548.
- Wettenhall, J.M., G.K. Smyth (2004) limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics (Oxford, England)* 20(18): 3705-3706.
- Wiedemann, M., B. Trueb (2000) Characterization of a novel protein (FGFRL1) from human cartilage related to FGF receptors. *Genomics* 69(2): 275-279.
- Wilkie, A.O. (1997) Craniosynostosis: genes and mechanisms. *Human molecular genetics* 6(10): 1647-1656.
- Wilkie, A.O., J.C. Byren, J.A. Hurst, J. Jayamohan, D. Johnson, S.J. Knight, T. Lester, P.G. Richards, S.R. Twigg, S.A. Wall (2010) Prevalence and complications of single-gene and chromosomal disorders in craniosynostosis. *Pediatrics* 126(2): e391-400.
- Wilkie, A.O., S.J. Patey, S.H. Kan, A.M. van den Ouweland, B.C. Hamel (2002) FGFs, their receptors, and human limb malformations: clinical and molecular correlations. *American journal of medical genetics* 112(3): 266-278.
- Wilkie, A.O., S.F. Slaney, M. Oldridge, M.D. Poole, G.J. Ashworth, A.D. Hockley, R.D. Hayward, D.J. David, L.J. Pulleyn, P. Rutland, et al. (1995) Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nature genetics* 9(2): 165-172.
- Wynn, T.A. (2008) Cellular and molecular mechanisms of fibrosis. *J Pathol* 214(2): 199-210.
- Xu, J., Z. Liu, D.M. Ornitz (2000) Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures. *Development (Cambridge, England)* 127(9): 1833-1843.
- Yacubian-Fernandes, A., A. Palhares, A. Giglio, R.C. Gabarra, S. Zanini, L. Portela, J.P. Plese (2004) Apert syndrome: analysis of associated brain malformations and conformational changes determined by surgical treatment. *Journal of neuroradiology* 31(2): 116-122.
- Yamaguchi, K., K. Ogita, S. Nakamura, Y. Nishizuka (1995) The protein kinase C isoforms leading to MAP-kinase activation in CHO cells. *Biochem Biophys Res Commun* 210(3): 639-647.
- Yang, F., Y. Wang, Z. Zhang, B. Hsu, E.W. Jabs, J.H. Elisseeff (2008) The study of abnormal bone development

- in the Apert syndrome Fgfr2+/S252W mouse using a 3D hydrogel culture model. *Bone* 43(1): 55-63.
- Yin, L., X. Du, C. Li, X. Xu, Z. Chen, N. Su, L. Zhao, H. Qi, F. Li, J. Xue, J. Yang, M. Jin, C. Deng, L. Chen (2008) A Pro253Arg mutation in fibroblast growth factor receptor 2 (Fgfr2) causes skeleton malformation mimicking human Apert syndrome by affecting both chondrogenesis and osteogenesis. *Bone* 42(4): 631-643.
- Yu J, Wjasow C, Backer JM (1998) Regulation of the p85/p110alpha phosphatidylinositol 3'-kinase. Distinct roles for the n-terminal and c-terminal SH2 domains. *J Biol Chem*. 273(46):30199-203.
- Yu, K., A.B. Herr, G. Waksman, D.M. Ornitz (2000) Loss of fibroblast growth factor receptor 2 ligand-binding specificity in Apert syndrome. *Proceedings of the National Academy of Sciences of the United States of America* 97(26): 14536-14541.
- Yu, K., J. Xu, Z. Liu, D. Sasic, J. Shao, E.N. Olson, D.A. Towler, D.M. Ornitz (2003) Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. *Development (Cambridge, England)* 130(13): 3063-3074.
- Zechel, S., S. Werner, K. Unsicker, O. von Bohlen und Halbach (2010) Expression and functions of fibroblast growth factor 2 (FGF-2) in hippocampal formation. *Neuroscientist* 16(4): 357-373.
- Zeisberg, M., E.G. Neilson (2009) Biomarkers for epithelial-mesenchymal transitions. *The Journal of clinical investigation* 119(6): 1429-1437.
- Zhang, C., L. Fu, J. Fu, L. Hu, H. Yang, T.H. Rong, Y. Li, H. Liu, S.B. Fu, Y.X. Zeng, X.Y. Guan (2009) Fibroblast growth factor receptor 2-positive fibroblasts provide a suitable microenvironment for tumor development and progression in esophageal carcinoma. *Clin Cancer Res* 15(12): 4017-4027.
- Zollukofer, C.P., M.S. Ponce de Leon (2010) The evolution of hominin ontogenies. *Semin Cell Dev Biol* 21(4): 441-452.

X. Anexos

Anexo 1: Tabela IV.1.

Anexo 2: Apert p.Ser252Trp mutation in FGFR2 alters osteogenic potential and gene expression of cranial periosteal cells. Fanganiello RD, Sertié AL, Reis EM, Yeh E, Oliveira NA, Bueno DF, Kerkis I, Alonso N, Cavaleiro S, Matsushita H, Freitas R, Verjovski-Almeida S, Passos-Bueno MR. Mol Med. 2007 13(7-8):422-42.

Anexo 3: Genetics of craniosynostosis: genes, syndromes, mutations and genotype-phenotype correlations. Passos-Bueno MR, Sertié, Jehee FS, Fanganiello R, Yeh E. Front Oral Biol. 2008;12:107-43. Review.

Table IV.1 Differentially expressed transcripts in FGF2-, FGF10- or FGF19 treated wild-type and S252W fibroblasts compared to the same samples without treatment. In each treatment, transcripts are ordered by average Fold change ratio (treated vs. non-treated) of the replicates.

FGF2 treated WT fibroblasts				
Probe ID	Gene Symbol	Gene Description	Gene Accession	Average Fold Change
8178439	C6orf15	chromosome 6 open reading frame 15	NM_014070	-6.97
8125537	HLA-DMA	major histocompatibility complex, class II, DM alpha	NM_006120	-4.57
7953812	PHC1	polyhomeotic homolog 1 (Drosophila)	NM_004426	-4.02
7972979	LOC100132785	similar to C21orf99 protein	XM_001720214	-3.80
8043456	---	cdna:Genscan chromosome:GRCh37:2:89428168:89442591:-1	ENST00000312946	-3.73
7910948	---	ncrna:misc_RNA chromosome:GRCh37:1:240861620:240861729:1	ENST00000362371	-3.57
8176806	PRY	PTPN13-like, Y-linked	NM_004676	-3.29
7970404	TPTE2	transmembrane phosphoinositide 3-phosphatase and tensin homolog 2	NM_130785	-2.78
8065163	---	---	ENST00000364618	-2.77
8005223	---	ncrna:misc_RNA chromosome:GRCh37:17:16613373:16613673:1	ENST00000386961	-2.74
7981730	IGLJ3	immunoglobulin lambda joining 3	AB001736	-2.72
7994026	LOC23117	KIAA0220-like protein	BC094882	-2.66
8146921	RDH10	retinol dehydrogenase 10 (all-trans)	NM_172037	-2.39
8101992	SLC39A8	solute carrier family 39 (zinc transporter), member 8	NM_022154	-2.37
7956271	HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)	NM_003725	-2.36
8133023	ZNF479	zinc finger protein 479	NM_033273	-2.36
8132911	---	---	ENST00000388070	-2.13
8148059	DEPDC6	DEP domain containing 6	NM_022783	-2.10
8069689	ADAMT55	ADAM metalloproteinase with thrombospondin type 1 motif, 5 (aggrecanase-2)	NM_007038	-2.10
8088979	VGLL3	vestigial like 3 (Drosophila)	NM_016206	-2.05
8007605	---	---	ENST00000390891	-2.04
8037298	LOC100130904	similar to CD177 molecule	XR_037541	-2.03
8048749	KCNE4	potassium voltage-gated channel, Isk-related family, member 4	NM_080671	-2.02

8019964	ARHGAP28	Rho GTPase activating protein 28	NM_001010000	-1.97
7908861	OCR1	ovarian cancer-related protein 1	AF314543	-1.97
8151334	MSC	musculin (activated B-cell factor-1)	NM_005098	-1.94
7970954	DCLK1	doublecortin-like kinase 1	NM_004734	-1.94
7943998	NNMT	nicotinamide N-methyltransferase	NM_006169	-1.94
8016412	---	ncrna:snRNA chromosome:GRCh37:17:46166040:46166146:-1	ENST00000384227	-1.88
8122240	---	ncrna:rRNA chromosome:GRCh37:6:136951378:136951493:1	ENST00000390940	-1.86
7985034	LOC441728	similar to Golgin subfamily A member 6 (Golgin linked to PML) (Golgin-like protein)	XR_017329	-1.86
8044737	---	---	ENST00000385854	-1.86
8016259	LRRC37A2	leucine rich repeat containing 37, member A2	NM_001006607	1.42
8122703	---	ncrna:misc_RNA chromosome:GRCh37:6:149952573:149952685:1	ENST00000384562	1.69
8010184	SEPT9	septin 9	AK093770	1.76
8121416	---	ncrna:misc_RNA chromosome:GRCh37:6:109626697:109626798:1	ENST00000364153	1.77
8089082	DCBLD2	discoidin, CUB and LCCL domain containing 2	NM_080927	1.79
8160431	LOC554202	hypothetical LOC554202	AK124391	1.79
8043945	MAP4K4	mitogen-activated protein kinase kinase kinase 4	NM_145686	1.81
8124391	HIST1H2AB	histone cluster 1, H2ab	NM_003513	1.81
7939365	FJX1	four jointed box 1 (Drosophila)	NM_014344	1.90
7939215	C11orf41	chromosome 11 open reading frame 41	NM_012194	1.91
8027352	---	---	uc002nnp.1	1.92
8143127	UNQ1940	HWKM1940	AK290250	1.96
7932826	KIAA1462	KIAA1462	NM_020848	1.97
8136786	---	ncrna:rRNA chromosome:GRCh37:9:33656498:33656965:1	ENST00000387929	1.97
7909568	DTL	denticleless homolog (Drosophila)	NM_016448	1.99
7929438	HELLS	helicase, lymphoid-specific	NM_018063	2.01
8097628	HHIP	hedgehog interacting protein	NM_022475	2.02
8143766	ARP11	actin-related Arp11	AB039791	2.02
8083094	RASA2	RAS p21 protein activator 2	NM_006506	2.08
7910022	CNIH3	cornichon homolog 3 (Drosophila)	NM_152495	2.09
7973067	NP	nucleoside phosphorylase	NM_000270	2.15
7974366	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	NM_000956	2.29

8151871	CCNE2	cyclin E2	NM_057749	2.32
8140668	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	NM_006080	2.33
7933469	ARHGAP22	Rho GTPase activating protein 22	NM_021226	2.34
8140864	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	NM_000786	2.37
8180257	CEP170	Homo sapiens centrosomal protein 170kDa (CEP170), transcript variant gamma, mRNA	NM_001042405.1	2.41
8086517	CDCP1	CUB domain containing protein 1	NM_022842	2.44
7973082	---	ncrna:misc_RNA chromosome:GRCh37:14:21147570:21147671:1	ENST00000362591	2.45
7965335	DUSP6	dual specificity phosphatase 6	NM_001946	2.47
8037374	PLAUR	plasminogen activator, urokinase receptor	NM_002659	2.50
8044499	SLC20A1	solute carrier family 20 (phosphate transporter), member 1	NM_005415	2.52
8174598	IL13RA2	interleukin 13 receptor, alpha 2	NM_000640	2.53
8005679	CCDC144C	coiled-coil domain containing 144C	BC036241	2.58
7964733	LOC204010	similar to 40S ribosomal protein SA (P40) (34/67 kDa laminin receptor)	BC107865	2.61
8054344	FLJ42986	FLJ42986 protein	AK124976	2.63
8043932	RPL31	ribosomal protein L31	NM_001099693	2.64
8141016	TFPI2	tissue factor pathway inhibitor 2	NM_006528	2.75
8094870	SHISA3	shisa homolog 3 (Xenopus laevis)	BC127690	2.82
7925525	CEP170	centrosomal protein 170kDa	NM_014812	2.87
8091411	TM4SF1	transmembrane 4 L six family member 1	NM_014220	2.87
8152617	HAS2	hyaluronan synthase 2	NM_005328	2.91
8149825	STC1	stanniocalcin 1	NM_003155	3.11
7992893	---	---	ENST00000386778	3.12
8105267	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	NM_002203	3.12
8106025	BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	NM_018429	3.86
8112045	ESM1	endothelial cell-specific molecule 1	NM_007036	4.35

FGF10 treated WT fibroblasts

Probe ID	Gene Symbol	Gene Description	Gene Accession	Average Fold Change
7981728	LOC100290036	similar to hCG2038926	/// ENST00000390601	-4.15
8116571	---	cdna:Genscan	GENSCAN0000003521	-4.05

		chromosome:GRCh37:6:583776:602526:1	3	
7916491	---	---	ENST00000364060	-3.74
8110417	LOC653316	hypothetical protein LOC653316	NM_001079527	-3.30
8010184	SEPT9	septin 9	AK093770	-3.10
8019709	WDR74	WD repeat domain 74	AK292330	-3.08
7972979	LOC100132785	similar to C21orf99 protein	XM_001720214	-3.06
8043363	C2orf59	chromosome 2 open reading frame 59	BC010491	-2.99
8045088	---	ncrna:misc_RNA chromosome:GRCh37:2:128825374:128825475:1	ENST00000363562	-2.98
8081546	---	ncrna:misc_RNA chromosome:GRCh37:3:110445868:110445968:1	ENST00000364556	-2.87
7999362	---	ncrna:rRNA chromosome:GRCh37:16:9658247:9658354:-1	ENST00000363598	-2.72
8117018	---	ncrna:snRNA chromosome:GRCh37:6:15315151:15315257:1	ENST00000364497	-2.66
8180329	UBE2D	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) (UBE2D3), transcript variant 1, mRNA	NM_003340.4	-2.66
8124846	---	ncrna:misc_RNA chromosome:GRCh37:6:30704081:30704191:-1	ENST00000363049	-2.63
8079163	---	---	ENST00000387758	-2.55
8000501	LOC440350	similar to nuclear pore complex interacting protein	NM_001018122	-2.54
8155554	AQP7P1	aquaporin 7 pseudogene 1	NR_002817	-2.51
7929541	CC2D2B	coiled-coil and C2 domain containing 2B	NM_001001732	-2.45
7919761	---	---	ENST00000385919	-2.40
8003230	DKFZp434O0320	hypothetical protein DKFZp434O0320	AL137562	-2.33
8091998	---	ncrna:snRNA chromosome:GRCh37:3:169437975:169438081:-1	ENST00000384050	-2.28
8007883	---	ncrna:misc_RNA chromosome:GRCh37:17:43603820:43604112:-1	ENST00000387975	-2.27
8104461	---	---	ENST00000363495	-2.26
7994265	---	ncrna:rRNA chromosome:GRCh37:16:26040233:26040346:1	ENST00000363059	-2.22
8118345	CFB	complement factor B	NM_001710	-2.11
8005953	SNORD4A	small nucleolar RNA, C/D box 4A	NR_000010	-1.93
8007794	ARL17	ADP-ribosylation factor-like 17 (ARL17), mRNA	ENST00000336125	-1.90
7934979	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	NM_014391	-1.87
7959482	---	ncrna:misc_RNA chromosome:GRCh37:12:123252646:123252747:	ENST00000384187	-0.30

		1		
8169156	---	ncrna:misc_RNA chromosome:GRCh37:X:105669798:105669899:1	ENST00000365537	1.85
8003812	OR3A3	olfactory receptor, family 3, subfamily A, member 3	NM_012373	1.85
7982955	---	---	ENST00000387772	1.87
8111666	---	ncrna:snRNA chromosome:GRCh37:5:37620087:37620190:-1	ENST00000365422	1.98
7962479	---	ncrna:misc_RNA chromosome:GRCh37:12:45274651:45274752:-1	ENST00000364918	2.03
7899346	CCDC72	coiled-coil domain containing 72	NM_015933	2.14
7973082	---	ncrna:misc_RNA chromosome:GRCh37:14:21147570:21147671:1	ENST00000362591	2.27
7987163	FMN1	formin 1	ENST00000320930	2.39
8007850	LRRC37A	leucine rich repeat containing 37A	NM_014834	2.43
7953812	PHC1	polyhomeotic homolog 1 (Drosophila)	NM_004426	2.60
8054800	---	---	ENST00000387490	2.74
7927097	LOC644773	ARP3 actin-related protein 3 homolog B pseudogene	XR_016836	2.97
7986598	LOC283767	FLJ40198 protein	NM_001001413	3.86
8132962	SNORA15	small nucleolar RNA, H/ACA box 15	NR_002957	4.00
7992893	---	---	ENST00000386778	5.33
8132690	---	ncrna:snRNA chromosome:GRCh37:19:223158:223261:-1	ENST00000386817	6.84

FGF19 treated WT fibroblasts

ProbeID	Gene Symbol	Gene Description	Gene Accession	Average Fold Change
8112469	SMA4	glucuronidase, beta pseudogene	X83300	-7.21
8117900	DDR1	discoidin domain receptor family, member 1 (DDR1), transcript variant 3, mRNA	ENST00000376575	-4.80
8118548	HLA-DRA	major histocompatibility complex, class II, DR alpha	NM_019111	-4.29
7991665	---	cdna:pseudogene chromosome:GRCh37:19:104601:105256:1	ENST00000328113	-4.24
8066210	---	---	ENST00000362637	-4.21
8045159	---	---	ENST00000385673	-3.48
8144388	FAM90A1	family with sequence similarity 90, member A1	NM_018088	-3.02
7927097	LOC644773	ARP3 actin-related protein 3 homolog B pseudogene	XR_016836	-2.99

7903414	AMY2A	amylase, alpha 2A (pancreatic)	NM_000699	-2.91
8165765	CXYorf2	chromosome X and Y open reading frame 2	AF193043	-2.89
8068662	---	ncrna:snoRNA chromosome:GRCh37:18:7995568:7995782:1	ENST00000387278	-2.89
8097459	---	---	ENST00000386235	-2.81
8076415	RNU13P1	RNA, U13 small nuclear pseudogene 1	X58061	-2.79
7972175	---	ncrna:misc_RNA chromosome:GRCh37:13:78929362:78929463:-1	ENST00000363816	-2.73
8133034	---	---	ENST00000386778	-2.68
7969339	---	ncrna:rRNA chromosome:GRCh37:13:58565484:58565598:1 gene:ENSG00000202422	ENST00000365552	-2.59
8016429	---	ncrna:snRNA chromosome:GRCh37:17:46274042:46274148:-1	ENST00000384576	-2.44
7999362	---	ncrna:rRNA chromosome:GRCh37:16:9658247:9658354:-1	ENST00000363598	-2.41
8156261	---	---	ENST00000388631	-2.37
7962479	---	ncrna:misc_RNA chromosome:GRCh37:12:45274651:45274752:-1	ENST00000364918	-2.32
7901361	---	---	ENST00000387793	-2.29
8005221	---	---	ENST00000386949	-2.10
8179935	TNXB	tenascin XB	NM_032470	-2.04
8125537	HLA-DMA	major histocompatibility complex, class II, DM alpha	NM_006120	-2.03
8136786	---	ncrna:rRNA chromosome:GRCh37:9:33656498:33656965:1	ENST00000387929	-1.41
8174226	---	ncrna:misc_RNA chromosome:GRCh37:X:102411048:102411139:-1	ENST00000363792	-1.39
8129309	---	ncrna:misc_RNA chromosome:GRCh37:6:121699943:121700044:-1	ENST00000384672	-1.01
8132830	---	ncrna:snRNA chromosome:GRCh37:12:51556684:51556790:1	ENST00000362954	1.58
8124967	BAT3	HLA-B associated transcript 3	NM_004639	1.85
8112914	---	---	ENST00000386720	1.92
8180318	TGIF	Homo sapiens TGFB-induced factor (TALE family homeobox) (TGIF), transcript variant 7, mRNA	NM_173211.1	1.97
7981737	LOC100291056	similar to hCG2036739	ENST00000390624	2.06
8053427	---	ncrna:misc_RNA chromosome:GRCh37:2:85687267:85687375:-1	ENST00000363066	2.11
8121214	---	ncrna:misc_RNA	ENST00000384290	2.19

		chromosome:GRCh37:6:99973973:99974081:1		
7948904	SNORD28	small nucleolar RNA, C/D box 28	NR_002562	2.21
8037231	PSG3	pregnancy specific beta-1-glycoprotein 3	BC005924	2.26
7953812	PHC1	polyhomeotic homolog 1 (Drosophila)	NM_004426	2.29
8115443	---	---	ENST00000354652	2.59
8007794	ARL17	ADP-ribosylation factor-like 17 (ARL17), mRNA	ENST00000336125	3.24
7984662	GOLGA	golgin-like protein	NM_018652	3.43
7962146	FAM60A	family with sequence similarity 60, member A	BC000024	3.47
7914127	IFI6	interferon, alpha-inducible protein 6	NM_002038	3.51
8007850	LRRC37A	leucine rich repeat containing 37A	NM_014834	3.74
8065325	---	cdna:Genscan chromosome:GRCh37:20:21147048:21147236:-1	GENSCAN0000001191 0	3.92
7992893	---	---	ENST00000386778	6.32

FGF2 treated S252W fibroblasts

Probe ID	Gene Symbol	Gene Description	Gene Accession	Average Fold Change
8180291	CDC2L1	Homo sapiens cell division cycle 2-like 1 (PITSLRE proteins) (CDC2L1), transcript variant 5, mRNA	NM_033489.1	-5.58
8027352	---	---	uc002nnp.1	-5.02
8118324	C2	complement component 2	NM_000063	-3.87
7952750	---	---	ENST00000387663	-3.67
8139776	---	---	ENST00000388162	-3.56
8031027	MIR520B	microRNA 520b	hsa-mir-520b	-2.83
8044351	LOC645231	similar to 60S ribosomal protein L22 (Heparin-binding protein HBp15)	XM_928270	-2.60
8043480	LOC100132426	similar to hCG1742442 /// similar to hCG1742442 /// similar to hCG1742442 /// hypothetical LOC100133034 /// similar to hCG1742442	XM_001715084	-2.47
8124967	BAT3	HLA-B associated transcript 3	NM_004639	-2.37
7898677	HS6ST1	heparan sulfate 6-O-sulfotransferase 1	NM_004807	-2.30
8106025	BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	NM_018429	-2.25
7919854	---	ncrna:misc_RNA chromosome:GRCh37:1:150854927:150855011:-1	ENST00000391210	-2.17
8058342	---	---	ENST00000385402	-2.16
8099240	---	cdna:pseudogene	ENST00000359859	-2.06

		chromosome:GRCh37:4:6872264:6872553:-1		
7976814	SNORD114-2	small nucleolar RNA, C/D box 114-2	NR_003194	-1.97
8140864	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	NM_000786	-1.94
8014437	TBC1D3G	TBC1 domain family, member 3G	NM_001040282	-1.92
8102160	LOC402182	hypothetical LOC402182	XR_016669	-1.88
8055236	LOC646743	similar to testicular serine protease 2	AY726562	-1.82
8069174	C21orf70	chromosome 21 open reading frame 70	AF391113	-1.72
7902541	IFI44L	interferon-induced protein 44-like	AL832618	-1.66
8040080	RSAD2	radical S-adenosyl methionine domain containing 2	NM_080657	-0.20
7968563	RFC3	replication factor C (activator 1) 3, 38kDa	NM_002915	1.55
8031152	RPS9	ribosomal protein S9	NM_001013	1.75
8148964	SEPT14	septin 14	BC131690	1.81
7933469	ARHGAP22	Rho GTPase activating protein 22	NM_021226	1.82
7917739	---	---	ENST00000390916	1.87
7998898	CLDN6	claudin 6	NM_021195	1.93
8116952	---	ncrna:snRNA chromosome:GRCh37:6:13214288:13214451:1	ENST00000383975	1.95
8142763	NAG13	Homo sapiens NAG13 (NAG13) mRNA, complete cds.	AF194537	1.97
8055596	---	---	ENST00000385525	1.98
7988132	STRC	stereocilin	NM_153700	1.98
7996569	PP10897	Homo sapiens PP10897 mRNA, complete cds.	AF370400	2.01
7959012	---	ncrna:misc_RNA chromosome:GRCh37:12:116155523:116155816:1	ENST00000386460	2.05
7940421	PGA3	pepsinogen 3, group I (pepsinogen A)	NM_001079807	2.09
8007850	LRRC37A	leucine rich repeat containing 37A	NM_014834	2.12
8025893	---	ncrna:misc_RNA chromosome:GRCh37:19:11519448:11519556:1	ENST00000365487	2.16
8003605	---	cdna:Genscan chromosome:GRCh37:17:56289:80140:1	GENSCAN0000000663 4	2.16
8102787	---	ncrna:snRNA chromosome:GRCh37:4:136917084:136917247:-1	ENST00000384592	2.41
7911341	TRNY	product=tRNA-Tyr	NC_001807	2.41
7958842	---	ncrna:misc_RNA chromosome:GRCh37:12:112587164:112587276:1	ENST00000363265	2.72

8013987	---	ncrna:misc_RNA chromosome:GRCh37:17:28386070:28386165:-1	ENST00000384284	2.72
8177947	TCF19	transcription factor 19 (SC1)	NM_007109	2.75
8166872	---	ncrna:rRNA chromosome:GRCh37:X:41152051:41152169:1	ENST00000362694	2.77
8015757	---	ncrna:misc_RNA chromosome:GRCh37:17:41149933:41150024:-1	ENST00000363257	2.79
8073680	---	---	ENST00000386848	2.89
8054800	---	---	ENST00000387490	2.95
8125042	LY6G6C	lymphocyte antigen 6 complex, locus G6C	AY359119	3.01
7948894	WDR74	WD repeat domain 74	AK292330	3.16
8056792	---	ncrna:misc_RNA chromosome:GRCh37:2:173422879:173422980:-1	ENST00000362996	3.32
7972561	---	ncrna:misc_RNA chromosome:GRCh37:13:100188573:100188650:-1	ENST00000390895	3.32
8176806	PRY	PTPN13-like, Y-linked	NM_004676	3.36
7914807	---	---	---	3.61
7984620	---	ncrna:rRNA chromosome:GRCh37:15:72150911:72151027:1	ENST00000364003	4.36
8019709	WDR74	WD repeat domain 74 /// RNA, U2 small nuclear /// RNA, U2B small nuclear	AK292330	4.37

FGF10 treated S252W fibroblasts
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Probe ID	Gene Symbol	Gene Description	Gene Accession	Average Fold Change
8178439	C6orf15	chromosome 6 open reading frame 15	NM_014070	-6.73
7986598	LOC283767	FLJ40198 protein	NM_001001413	-4.96
8005689	LOC220594	TL132 protein	NR_003554	-3.99
7920201	SPRR2B	small proline-rich protein 2B	NM_001017418	-3.75
8054800	---	---	ENST00000387490	-3.26
8133314	NCF1	neutrophil cytosolic factor 1, (chronic granulomatous disease, autosomal 1)	NM_000265	-3.25
8149361	FAM90A1	family with sequence similarity 90, member A1	NM_018088	-2.48
7981895	GOLGA8E	golgi autoantigen, golgin subfamily a, 8E	NM_001012423	-2.34
8037231	PSG3	pregnancy specific beta-1-glycoprotein 3	BC005924	-2.26
7919761	---	---	ENST00000385919	-2.16
8084878	---	ncrna:misc_RNA chromosome:GRCh37:3:193341978:193342090:1	ENST00000384640	-2.06

8132830	---	ncrna:snRNA chromosome:GRCh37:12:51556684:51556790:1	ENST00000362954	-2.04
8156253	FAM75C1	family with sequence similarity 75, member C1	AK093374	-2.01
7969574	KRT18P27	keratin 27 pseudogene 27	XR_016173	-2.00
7904959	---	---	ENST00000386101	-1.97
7907156	XCL1	chemokine (C motif) ligand 1	NM_002995	-1.95
7904048	---	ncrna:snoRNA chromosome:GRCh37:1:113195210:113195310:1 gene:ENSG00000238975	ENST00000386572	-1.84
7948148	OR5M10	olfactory receptor, family 5, subfamily M, member 10	NM_001004741	-1.83
8180318	TGIF	Homo sapiens TGFB-induced factor (TALE family homeobox) (TGIF), transcript variant 7, mRNA	NM_173211.1	-1.80
8049123	ALPP	alkaline phosphatase, placental (Regan isozyme)	NM_001632	-1.78
8135031	MUC12	mucin 12, cell surface associated	AF147791	-1.69
8100941	---	---	ENST00000388112	-1.64
8180341	RAC1	Homo sapiens ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1), transcript variant Rac1b, mRNA	NM_018890.2	1.52
8124385	HIST1H4B	histone cluster 1, H4b	NM_003544	1.55
8124531	HIST1H3I	histone cluster 1, H3i	NM_003533	1.60
8112469	SMA4	glucuronidase, beta pseudogene	X83300	1.60
7934731	LOC642538	similar to nuclear DNA-binding protein	XM_926027	1.69
8035819	ZNF208	zinc finger protein 208	ENST00000340708	1.69
8124645	OR12D3	olfactory receptor, family 12, subfamily D, member 3	NM_030959	1.76
8176234	CLIC2	chloride intracellular channel 2	NM_001289	1.78
7904930	GPR89B	G protein-coupled receptor 89B	NM_016334	1.81
7961514	MGP	matrix Gla protein	NM_000900	1.94
8074867	DKFZP434P211	POM121-like protein	AK292412	1.99
8165672	RFC1	replication factor C (activator 1) 1, 145kDa	NC_001807	1.99
8176482	---	cdna:Genscan chromosome:GRCh37:Y:8759781:8783985:	ENST00000311828	2.02
7948894	WDR74	WD repeat domain 74	AK292330	2.07
8047441	---	ncrna:misc_RNA chromosome:GRCh37:2:202288236:202288327:1	ENST00000365138	2.15
8083592	---	ncrna:misc_RNA chromosome:GRCh37:3:156871337:156871429:1	ENST00000364908	2.18
8117748	MOG	myelin oligodendrocyte glycoprotein	BC035938	2.26

8053785	LOC120126	hypothetical protein	NM_001101321	2.31
8118634	RING1	ring finger protein 1	NM_002931	2.35
7981874	GOLGA8E	golgi autoantigen, golgin subfamily a, 8E	NM_001012423	2.36
7911283	OR2T3	olfactory receptor, family 2, subfamily T, member 3	NM_001005495	2.43
7914180	SPCS2	signal peptidase complex subunit 2 homolog (S. cerevisiae)	NM_014752	2.50
8125537	HLA-DMA	major histocompatibility complex, class II, DM alpha	NM_006120	2.53
8125007	LY6G5C	lymphocyte antigen 6 complex, locus G5C	NM_025262	2.57
8130464	C6orf35	chromosome 6 open reading frame 35	ENST00000397906	2.60
7962146	FAM60A	family with sequence similarity 60, member A	BC000024	2.62
7908488	CFHR1	complement factor H-related 1	NM_002113	2.67
7981728	---	cdna:known chromosome:GRCh37:16:33629702:33630420:1	ENST00000390598 /// ENST00000390601	2.78
8133654	WBSCR19	Williams Beuren syndrome chromosome region 19	NM_175064	2.79
7972561	LOC100290036	similar to hCG2038926	ENST00000390895	2.79
7972979	LOC100132785	similar to C21orf99 protein	XM_001720214	3.01
8166872	---	ncrna:rRNA chromosome:GRCh37:X:41152051:41152169:1	ENST00000362694	3.03
8074714	DKFZP434P211	POM121-like protein	NR_003714	3.22
8161537	CBWD3	COBW domain containing 3	NM_201453	3.31
8017173	TBC1D3P2	TBC1 domain family, member 3 pseudogene 2	BC058890	3.62
8177947	TCF19	transcription factor 19 (SC1)	NM_007109	3.97
7981722	IGHA1	immunoglobulin heavy constant alpha 1	AK128476	4.81

FGF19 treated S252W fibroblasts				
Probe ID	Gene Symbol	Gene Description	Gene Accession	Average Fold Change
8027352	---	---	uc002nnp.1	-4.91
8178439	C6orf15	chromosome 6 open reading frame 15	NM_014070	-4.04
7927513	FAM21A	family with sequence similarity 21, member A	AK055424	-3.43
8132501	---	ncrna:snRNA chromosome:GRCh37:12:112002625:112002687:-1	ENST00000459067	-3.14
8008627	NOG	noggin	NM_005450	-2.43
8133654	WBSCR19	Williams Beuren syndrome chromosome region 19	NM_175064	-2.38

7976814	SNORD114-2	small nucleolar RNA, C/D box 114-2	NR_003194	-2.27
8176806	PRY	PTPN13-like, Y-linked	NM_004676	-2.23
8117018	---	ncrna:snRNA chromosome:GRCh37:6:15315151:15315257:1	ENST00000364497	-2.14
8156290	CKS2	CDC28 protein kinase regulatory subunit 2	NM_001827	-2.14
8045208	FKSG30	kappa-actin	ENST00000329081	-2.11
8135929	---	ncrna:snRNA chromosome:GRCh37:19:223158:223261:-1	ENST00000386817	-1.98
8155528	---	---	ENST00000388224	-1.86
8156253	FAM75C1	family with sequence similarity 75, member C1	AK093374	-1.79
8092241	KCNMB3	potassium large conductance calcium-activated channel, subfamily M beta member 3	NM_171830	1.67
7933638	FLJ31958	hypothetical protein FLJ31958	AK056520	1.76
8141533	---	ncrna:misc_RNA chromosome:GRCh37:7:100127986:100128282:-1	ENST00000386086	1.84
7938329	SNORA23	small nucleolar RNA, H/ACA box 23	NR_002962	1.86
8133770	CCDC146	coiled-coil domain containing 146	NM_020879	1.92
7962479	---	ncrna:misc_RNA chromosome:GRCh37:12:45274651:45274752:-1	ENST00000364918	1.95
8066117	SAMHD1	SAM domain and HD domain 1	NM_015474	2.04
7987572	---	---	ENST00000387750	2.07
8031027	MIR520B	microRNA 520b	hsa-mir-520b	2.07
8127396	LOC100134271	similar to OTTHUMP00000016679	ENST00000370621	2.08
7920256	---	ncrna:misc_RNA chromosome:GRCh37:1:153472943:153473240:-1	ENST00000391165	2.08
7960359	RPS27	ribosomal protein S27	ENST00000447487	2.10
8152759	TATDN1	TatD DNase domain containing 1	NM_032026	2.32
8166872	---	ncrna:rRNA chromosome:GRCh37:X:41152051:41152169:1	ENST00000362694	2.45
7958895	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	NM_006187	2.67
8133070	---	ncrna:snoRNA chromosome:GRCh37:7:64526377:64526510:1	ENST00000384614	2.70
7988132	STRC	stereocilin	NM_153700	2.73
8082100	PARP14	poly (ADP-ribose) polymerase family, member 14	NM_017554	2.84
8155574	ANKRD20A1	ankyrin repeat domain 20 family, member A1	NM_032250	2.85
7953844	DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, <i>S. cerevisiae</i>)	NM_030653	2.91

7902553	IFI44	interferon-induced protein 44	NM_006417	2.93
8133074	FLJ25037	hypothetical protein FLJ25037	AK057766	3.12
7977456	A26C2	ANKRD26-like family C, member 2	NM_001005356	3.14
7992893	---	---	ENST00000386778	3.29
7981728	LOC100290036	similar to hCG2038926	ENST00000390598	3.30
8160559	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	NM_014314	3.32
8074714	DKFZP434P211	POM121-like protein	NR_003714	3.56
7899346	CCDC72	coiled-coil domain containing 72	NM_015933	3.67
8176482	---	cdna:Genscan chromosome:GRCh37:Y:8759781:8783985:1	ENST00000311828	3.73
8040080	RSAD2	radical S-adenosyl methionine domain containing 2	NM_080657	4.37
8000636	LOC440350	similar to nuclear pore complex interacting protein	NM_001018122	4.49
7902541	IFI44L	interferon-induced protein 44-like	AL832618	5.47

Apert p.Ser252Trp Mutation in FGFR2 Alters Osteogenic Potential and Gene Expression of Cranial Periosteal Cells

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Apert syndrome (AS), a severe form of craniosynostosis, is caused by dominant gain-of-function mutations in FGFR2. Because the periosteum contribution to AS cranial pathophysiology is unknown, we tested the osteogenic potential of AS periosteal cells (p.Ser252Trp mutation) and observed that these cells are more committed toward the osteoblast lineage. To delineate the gene expression profile involved in this abnormal behavior, we performed a global gene expression analysis of coronal suture periosteal cells from seven AS patients (p.Ser252Trp), and matched controls. We identified 263 genes with significantly altered expression in AS samples (118 upregulated, 145 downregulated; SNR ≥ 10.41 , $P \leq 0.05$). Several upregulated genes are involved in positive regulation of cell proliferation and nucleotide metabolism, whereas several downregulated genes are involved in inhibition of cell proliferation, gene expression regulation, cell adhesion, and extracellular matrix organization, and in PIK3-MAPK cascades. AS expression profile was confirmed through real-time PCR of a selected set of genes using RNAs from AS and control cells as well as from control cells treated with high FGF2 concentration, and through the analysis of genes involved in FGF-FGFR signaling. Our results allowed us to: (a) suggest that AS periosteal cells present enhanced osteogenic potential, (b) unravel a specific gene expression signature characteristic of AS periosteal cells which may be associated with their osteogenic commitment, (c) identify a set of novel genes involved in the pathophysiology of AS or other craniosynostotic conditions, and (d) suggest for the first time that the periosteum might be involved in the pathophysiology of AS.

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INTRODUCTION

Craniosynostosis, the premature fusion of one or more cranial sutures, is a relatively common malformation with an incidence of 1:2,500 births. Apert syndrome (AS [MIM 101200]) is one of the most severe forms of craniosynostosis, accounting for 4.5% of all cases in different populations (1). AS also is characterized by mid-facial hypoplasia and severe symmetric bony and cutaneous syndactyly of the hands and feet. Although the coronal suture is closed at birth, the squamosal and the lambdoid suture are opened and the anterolateral fontanelles are much enlarged because of a wide midline calvarial

defect. Many other anomalies are associated with AS, such as central nervous system, cardiovascular, urogenital, and dermatologic abnormalities (2–5). Inheritance is autosomal dominant and most cases represent new mutations which are exclusively of paternal origin (6). Early corrective cranial surgical intervention is needed to allow proper brain and skull growth. As a result of continuous bone healing defect, several surgeries are usually necessary during childhood and puberty.

Two activating missense mutations on the fibroblast growth factor 2 receptor (FGFR2) cause the great majority of AS cases: p.Ser252Trp (c.755C > G) and

p.Pro253Arg (c.758C > G) (7). They are located in the linker region between immunoglobulin-like loops II and III of the FGFR2: the former is present in about two thirds of the patients and is associated with a more severe craniofacial phenotype and the latter is found in the remaining one third of the patients and is associated with a more severe syndactyly (4). These mutations are present in mesenchymal “FGFR2c” and epithelial “FGFR2b” splice isoforms and they both involve substitutions of bulky side-chain amino acids, which can alter the relative orientation of the ligand-binding sites of this receptor. Either of these changes lead to enhanced FGFR2 ligand binding affinity and decreased specificity (8–10).

FGFR activation by FGFs (fibroblast growth factors) can induce several different cell processes, such as differentiation, proliferation, migration, and apoptosis by

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activating a variety of intracellular pathways, including MAPK (mitogen-activating protein kinase), PI3K (Phosphoinositide-3 kinase), PKC (protein kinase C), and STAT (signal transducers and activator of transcription) pathways (5). Cellular context and cell nature are important factors that determine the cellular consequences of receptor stimulation. Normal FGFR1-3 signaling also is crucial in the processes of cell growth and differentiation at the cranial sutural margins and alterations in the molecular pathways or in the timing of the activation events can lead to the premature fusion of these margins.

Most of the studies on the cellular consequences of AS mutant FGFR2 activation have been focused on calvarial osteoblasts, both in vitro and in vivo (11–17). Several lines of evidence indicate that the periosteum plays an important role in cranial bone regeneration because removal of this tissue decreases vascularization and calcification of cranial defects in animal models (18,19). However, little or no information about the periosteal cells' role in the pathophysiology of AS is available. We have postulated that the AS cranial periosteal cells may behave abnormally and contribute to enhanced suture ossification, and therefore we decided in the present work to test this hypothesis.

A transcriptional gene expression signature in periosteal AS cells has been previously reported based on the analysis of only one patient harboring the p.Pro253Arg mutation and two controls (20). Therefore, the confirmation of these results with a larger number of patients and controls is clearly warranted. Nonetheless, given that one of the most considerable challenges in the field of molecular medicine is to dissect the mechanisms of isolated cases of genetic diseases, including syndromic craniosynostosis, the identification of gene expression profiles associated with specific Mendelian disorders could become a powerful tool to unravel the underlying causes of this group of diseases.

Thus, the present study aimed to evaluate if p.Ser252Trp FGFR2 mutant periosteal cells present a greater commitment toward osteogenic differentiation,

which could contribute to the pathophysiology of AS, and to address if these cells present a transcriptional signature that would be involved in the molecular mechanisms of this syndrome.

SUBJECTS, MATERIAL, AND METHODS

Subjects

During corrective surgery, overlying periosteum from the coronal suture region of seven AS patients (three males and four females aged from three months to 14 years) was meticulously dissected away from surrounding tissues to isolate intact periosteal flaps. Control periosteum was obtained using the same procedure from the coronal suture region of seven subjects (three males and four females aged from 11 months to 13 years) with no evidence of bone disease during craniotomy for removal of brain tumors. The project was approved by the local ethical committee and appropriate informed consent was obtained from each subject or their legal guardians.

The presence of the p.Ser252Trp mutation was confirmed by direct DNA sequencing.

Cell Culture and RNA Isolation

Primary periosteal fibroblast cells derived from the periosteal flaps were grown in fibroblast growth medium (80 percent DMEM, 20 percent fetal bovine serum [FSB] and 2 mmol/L l-glutamine, penicillin, and streptomycin), in a humidified incubator at 37°C and five percent CO₂. Cells were passaged at near confluence with trypsin-EDTA. All tests were performed between the sixth and the eighth subculture.

Although the primary periosteal fibroblast cells appeared as a homogeneous population of fibroblastoid cells, to further attest that the surgical isolation of periosteum as well as the cell culture expansion procedure were leading to a homogeneous cell sample, immunocytochemistry experiments were performed in two control cell lineages using antibodies specific for mesenchymal cells (SH2 and SH3) and epithelial cells (Cytokeratin 18

and Integrin B1). It was observed that these cells stained homogeneously for the mesenchymal cells markers but not for the epithelial ones. As a positive control, cultured skin fibroblasts from an unaffected subject were used, which stained homogeneously for the epithelial cells markers but not for the mesenchymal cells markers.

Total RNA was isolated from confluent control and AS cells using TRIZOL reagent (Gibco BRL Gaithersburg, MD, USA) and purified with RNeasy minicolumns (Qiagen Valencia, CA, USA). RNA quality and concentration were assessed respectively by 1.5 percent agarose gel electrophoresis and spectrophotometry.

FGFR2 Expression Analysis

To verify the presence of the FGFR2 in AS and control periosteal cells we performed RT-PCR, Western blot, and immunocytochemical experiments.

Expression Analysis of *FGFR2* by RT-PCR

One step RT-PCR (Invitrogen Carlsbad, CA, USA) was performed with 2 µg of total RNA samples from five AS patients and four control subjects and specific primer pairs for each of the two major isoforms of *FGFR2* (*FGFR2b* and *FGFR2c*). The same forward primer (*FGFR2*-6F: 5'-agtgtggtcccatctgacaag-3') was combined with a reverse primer specific for either the *FGFR2b* (*FGFR2b*-9R: 5'-ggcctgcctatataattgga-3') or *FGFR2c* (*FGFR2c*-10R: 5'-atagaattaccgcccaagcac-3') isoform. A total of 35 cycles of amplification were performed. Reaction products were resolved alongside a 100-bp ladder on 1.5 percent agarose gel.

Western Blot and Immunocytochemical Experiments

Cell lysates from three AS patients and from three control subjects were prepared in RIPA Buffer (5 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.1 percent SDS, 0.5 percent sodium deoxycholate, one percent Nonidet 40) and 500 µg of protein were subject to Western blot analysis using a dilution of 1:250 of primary antibody BEH (H-80: sc-20734,

Santa Cruz Biotechnology Santa Cruz, CA, USA), and 1:1000 of anti-rabbit alkaline-phosphatase conjugated secondary antibody. For the immunocytochemical experiments, cells were treated with paraformaldehyde (four percent during 30 min) and labeled with 1:100 of the first antibody BEH and 1:100 of the second anti-rabbit-Cy3 antibody. As positive control we used the murine adeno-cortical Y1 cells (kindly provided by Dr. Hugo Armelin). Control staining without primary antibody was used as negative control.

In Vitro Osteogenic Differentiation

To induce osteogenic differentiation, periosteal fibroblasts from two AS patients and two controls were cultured for three weeks in DMEM ten percent FBS, 0.1 mM dexamethasone, 50 mM ascorbate-2-phosphate, 10 mM β -glycerophosphate, 0.1 percent antibiotic, with media changes every three to four days. After 21 days, calcified matrix production was analyzed by von Kossa staining as previously described (21).

Microarray Assays, Normalization, and Statistical Analyses

Gene expression experiments were performed using CodeLink bioarray systems (GE Healthcare Buckinghamshire, UK) according to manufacturer's protocols. In brief, first-strand cDNA was produced using 2 μ g of total RNA from each sample, Superscript II reverse transcriptase and a T7-poly-dT primer. Second-strand cDNA was produced using RNase H and *E. coli* DNA polymerase I. Double-stranded cDNA was purified on a QIAquick column (Qiagen) and biotin-labeled cRNA targets were generated by an in vitro transcription reaction using T7 RNA polymerase and biotin-11-UTP (Perkin Elmer-Foster City, CA, USA). Fragmented cRNA from each sample was hybridized to CodeLink microarrays containing approximately 20,000 (20K, five samples) or 55,000 (55K, nine samples) 30-mer probes overnight at 37°C in a shaking incubator at 300 rpm. After post-hybridization washes, hybridized targets were revealed

by incubating the arrays with a Cy5-Streptavidin conjugate. The reagents used in the synthesis and fragmentation of cRNA were provided in the CodeLink expression assay kit (GE Healthcare). Signal of the Cy5-dye from hybridized targets were detected with a GenePix 4000B scanner (Axon Instruments Foster City, CA, USA). CodeLink Expression Analysis software (GE Healthcare) was used to obtain background-subtracted spot intensities from microarray images. A set of 19,683 cDNA probes present in both the 20K and 55K arrays were analyzed. From these, only 9,543 probes that had valid measurements (i.e. were detected above the average array background as determined by a set of negative controls represented in the CodeLink arrays) in at least six out of seven samples from AS or control samples were further analyzed. To make experiments comparable, intensity data from different hybridizations were normalized by two different methods: (i) trimmed mean excluding the 20 percent of spots with higher and lower intensities, or (ii) Lowess, local weighted scatter-plot smoothing (22). Data adjusted by Lowess to a reference file resulted in lower coefficient of variation values across all samples and were used in further analyses. To identify gene expression signatures of AS, a Signal-to-Noise Ratio (SNR) metric (23) was used to compare the expression intensity data from AS samples to control samples. The SNR parameter essentially is a measure of signal strength relative to background noise. The distance between the two groups was measured by a signal-(expression intensity) to-noise (variation) ratio. The signal-to-noise comparison gives an indication of the level of separation for the means of the two distributions defining the gene intensities of the two groups, and it was calculated as $SNR = \sqrt{(\mu_1 - \mu_2)^2 / (SD_1 + SD_2)}$, where μ_1 and μ_2 are the mean intensities of AS and control groups, respectively, and SD_1 and SD_2 the corresponding standard deviations. For each gene, higher absolute SNR values indicate a higher difference of expression between AS and control samples with a lower dispersion within each group. A

cutoff $SNR \geq |0.4|$ was used to select differentially expressed genes. Statistical significance of the differential expression (P values) was ascertained by bootstrap resampling, i.e. by re-calculating SNR values following 10,000 random permutations of sample labels and computing the frequency at which each SNR value measured in the original set was observed in the randomly permuted data (24). The robustness of identified AS gene expression signature was evaluated by sample leave-one-out cross-validation (25). Essentially, one sample is removed and a new set of significantly altered genes is determined using the remainder samples. This procedure was repeated for each one of the 14 AS/control samples and the frequency at which each gene appears in the various "leave-one-out" datasets at a given significance ($P \leq 0.05$) was annotated.

The reproducibility of the gene expression measurements was assessed by generating two independent replicate target preparations for each of two AS patients and one control and hybridization of the replicates to separate 55K or 20K microarrays, the two types of microarray platforms used in our gene expression experiments. Pair-wise comparison using the Pearson correlation was applied to compare the intensity values from the 19,683 probes common to both CodeLink platforms. Intensity values from the probes common to both microarray platforms were highly correlated between sample replicates (average Pearson correlation = 0.95 ± 0.01) among the three sets of hybridizations. This result confirms that intensity measurements obtained in the different platforms can be compared without any significant loss in accuracy. In addition, the average correlation measured in pairwise analyses with data from all 14 different samples was 0.83 ± 0.07 .

Reverse Transcription Reactions and Quantitative Real-time PCR

Complementary DNA (cDNA) was produced from four μ g of total RNA using Superscript II reverse transcription kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using approximately 200 ng

Table 1. Sequence of the primers used in the quantitative Real Time PCR experiments.

Gene	Forward sequence	Reverse sequence
<i>CENPN</i>	TCAGTGATGCTGCCCTGTTAGA	AGTGATGCTGCCCTGTTAGACA
<i>STMN1</i>	GGCAGGACTTTCCTTATCCCA	GGCAGGACTTTCCTTATCCCA
<i>SPAG5</i>	GAGITCAAGGAGGTGCTGAAGA	TGCTGGAGGTCCTGCTATTTC
<i>RRM2</i>	CTTTGTCATCTCCCCATCGAG	TGCTGAATGTCCTTGGAGAGGT
<i>HIP2</i>	GAGITCAAGGAGGTGCTGAAGA	TGCTGGAGGTCCTGCTATTTC
<i>EEF1B2</i>	TTCGGAGACCTGAAAAGCCCT	CGGCTTCAAATACTGCCACATC
<i>HPRT1</i>	TGACACTGGCAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
<i>SDHA</i>	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG

of cDNA and SYBR Green PCR master mix in an ABI Prism 7100 system (Applied Biosystems Foster City, CA, USA). The PCR conditions were: 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s for 40 cycles.

Samples from four AS and four controls were run in triplicates, and the threshold suggested by the instrument software was used to calculate Ct. To normalize the readings we used Ct values from *HPRT1* (*Hypoxanthine phosphoribosyltransferase 1*) and *SDHA* (*succinate dehydrogenase complex, subunit A*) as internal controls in each run, obtaining a delta Ct value for each tested gene (*STMN1*, *SPAG5*, *RRM2*, *HIP2*, *CENPN*, *EEF1B2*). Primers used in this study are summarized in Table 1 as supplementary information.

Exogenous FGF2 Treatment

Control periosteal fibroblasts were grown to about 80 percent confluence in six 25 cm² cell culture bottles as described. Cells were washed with PBS and then were serum starved for 24 h in DMEM not supplemented with FBS. After this period, control cells (three bottles) were treated with DMEM containing 0.5 percent FBS and experimental cells (three bottles) were treated with DMEM 0.5 percent FBS and recombinant bovine FGF2 (provided by Dr. Hugo Armelin) to a final concentration of 36 ng/mL (or 2000 pM – at this high concentration phosphorylation of both wild type and mutant FGFR2c was similar, as observed by 9). Control and experimental cells were harvested at three, six, and 24 h after addition of FGF2, and had its total RNA isolated and purified as described. cDNA was obtained by reverse

transcription of two µg of total RNA using Superscript II (Invitrogen). qRT-PCR was used to measure expression levels of *STMN1*, *SPAG5*, *RRM2*, *HIP2*, *CENPN*, *EEF1B2* after FGF2 stimulation.

RESULTS

Morphology and FGFR2 Expression Analysis in Periosteal Cells

Cultured coronal suture periosteal cells from wild type controls and AS pa-

tients appeared microscopically to be a homogeneous population of adherent fibroblast-like cells, which exhibited neither morphology alteration nor significant cell death after several passages.

As expected for a homogeneous cellular population of periosteal cells with mesenchymal origin, we observed only the expression of the *FGFR2c* isoform in control and AS periosteal cells, with no apparent difference between these two, as determined by RT-PCR. FGFR2 protein also was detected in similar amounts both in control and AS cells (data not shown).

Differentiation of Periosteal Fibroblasts to the Osteoblast Cell Lineage

To address if AS periosteal cells present a greater osteogenic potential, control and AS cells (Figure 1A) were treated with osteogenic induction medium. We found that the potentiality to differentiate to osteogenic lineage varied significantly between control and AS cellular

Figure 1. (A) Morphology of undifferentiated AS periosteal cells. (B and C) Osteogenic differentiation of periosteal cells after 21 days of culture in osteogenic medium. Secretion of a calcified extracellular matrix was clearly observed. (D) Control cells after 21 days of osteogenic induction without signals of osteogenic differentiation (mineralized matrix are absent). Differentiation was accessed by von Kossa staining.

Figure 2. Hierarchical clustering of a 263 gene expression signature of AS samples relative to normal control samples. Individual genes are represented in lines and different samples are represented in rows. Expression level of each gene is represented by the number of standard deviations above (red) or below (blue) the average value for that gene across all samples. Color intensity is proportional to the number of standard deviations in the range -1.5 to 1.5 , as indicated by the color-coded bar at the bottom of the figure.

populations. Four days after the beginning of treatment, an osteoblast-like phenotype was observed in AS cells, while control cells maintained the fibroblast-like morphology. After 21 days of treatment, we observed several areas positive for calcium staining by the von Kossa reaction in AS cells (Figure 1 B and C), but not in control cells (Figure 1 D).

Differential Gene Expression

To verify whether there is an expression signature associated with the presence of the p.Ser252Trp mutation, which could explain the altered mutant cell behavior, we performed genome-wide expression analysis with RNA samples from seven AS patients and seven gender- and age-matched controls using either 55K or 20K microarray platforms. We found 9,543 transcripts (out of 19,683 transcripts under analysis) that were expressed in at least six out of seven sam-

ples from AS or control subjects. The average expression levels of 263 genes were found to be significantly altered in AS samples when compared with normal subjects (118 genes upregulated and 145 downregulated) (Figure 2, Table 2 – supplementary information), using as thresholds an absolute $\text{SNR} \geq |0.4|$ and $P \leq 0.05$ (see Methods section for details).

Submitting the 263 differentially expressed genes to the KEGG Pathway Database, the Gene Ontology Database (AmiGO), and the NCBI (Gene) database, we found that 186 of them have a known or inferred function. Among them, we found 98 genes that could be grouped in the following functional categories: regulation of cell proliferation (36 genes; among them, 28 and eight genes are involved in positive and negative regulation of cell proliferation, respectively), nucleotide metabolism (ten genes), regulation of gene expression (32 genes),

apoptosis (17 genes), cell adhesion (13), extracellular matrix component or biogenesis (seven genes), and MAPK pathways (16 genes) (Table 3). Some genes belong to more than one functional category. These categories were selected because they contain the largest numbers of genes, and because genes with related biological functions already were associated to FGFR2 signaling.

From this analysis, we observed that the most abundant classes of transcripts were those associated with regulation of cell proliferation and regulation of gene expression. In addition, among the 98 genes functionally classified, 45 were upregulated while 53 were downregulated in AS cells. Among the upregulated genes, the majority belong to positive regulation of cell proliferation and nucleotide metabolism categories (29/45 genes or $\sim 64.4\%$) (Table 3).

Table 2. 263 transcripts differentially expressed in AS cells as compared with control samples. Transcripts are ordered by their Apert-to-control ratios (Log 2).

Gene (Official Symbol)	Gene name	Accession number	SNR ^a	P value	Apert-to- control Ratio (Log2)	x-fold down- regulated in Apert
<i>DKFZp434B1231^b</i>	eEF1A2 binding protein	NM_178275.3	-0.86	0.010	-2.66	6.31
<i>TNNT1^c</i>	troponin T type 1 (skeletal, slow)	F36108.1	-0.662	0.017	-2.45	5.46
<i>TNXB^b</i>	tenascin XB	NM_032470.2	-0.769	0.015	-2.32	4.98
<i>CYHR1^b</i>	cysteine/histidine-rich 1	AB007965.1	-1.013	0.010	-2.21	4.62
<i>RTN4RL1^b</i>	reticulon 4 receptor-like 1	AL834409.1	-0.812	0.023	-1.83	3.57
<i>CCRL1</i>	chemokine (C-C motif) receptor-like 1	NM_016557.2	-0.65	0.035	-1.77	3.42
<i>NISC_np07a06y1</i> <i>NICHD_</i> <i>HS_Ut1</i>	NISC_np07a06y1 NICHD_ HS_Ut1 cDNA clone IMAGE:5936938 5'	CB215524.1	-0.422	0.047	-1.76	3.39
<i>PCNXL2^b</i>	pecanex-like 2 (Drosophila)	NM_014801.2	-0.773	0.023	-1.75	3.36
<i>CFB</i>	complement factor B	NM_001710.3	-0.678	0.027	-1.74	3.34
<i>DDIT4^b</i>	DNA-damage-inducible transcript 4	NM_019058.1	-0.564	0.022	-1.71	3.28
<i>RARRES3^b</i>	retinoic acid receptor responder (tazarotene induced) 3	NM_004585.2	-0.773	0.022	-1.64	3.12
<i>ZC3H12A</i>	zinc finger CCCH-type containing 12A	NM_025079.1	-0.499	0.047	-1.62	3.08
<i>PIK3C2B^c</i>	phosphoinositide-3-kinase, class 2, β polypeptide	NM_002646.2	-0.903	0.004	-1.54	2.91
<i>TP53111</i>	tumor protein p53 inducible protein 11	BC045666.1	-0.702	0.026	-1.48	2.79
<i>GBP2</i>	guanylate binding protein 2, interferon-inducible	NM_004120.3	-0.689	0.031	-1.43	2.69
<i>ChGn^c</i>	chondroitin beta1,4 N-acetylgalactos-aminyltransferase	NM_018371.3	-0.819	0.015	-1.42	2.68
<i>ZNF385^b</i>	zinc finger protein 385	NM_015481.1	-0.955	0.011	-1.38	2.61
<i>UI-E-CQ1-aew-i-06-0-Ulr1</i> <i>UI-E-CQ1</i>	UI-E-CQ1-aew-i-06-0-Ulr1 UI-E-CQ1 cDNA clone UI-E-CQ1-aew-i-06-0-UI 5'	BM695626.1	-0.661	0.042	-1.37	2.59
<i>PDGFRA</i>	platelet-derived growth factor receptor, α polypeptide	NM_006206.2	-0.653	0.045	-1.34	2.53
<i>LRRC17^b</i>	leucine rich repeat containing 17	NM_005824.1	-0.643	0.039	-1.25	2.38
<i>yr27d04r1</i>	yr27d04r1 Soares fetal liver spleen 1NFLS cDNA clone IMAGE:206503 5'	H59349.1	-0.708	0.039	-1.25	2.38
<i>MGC:71335^b</i>	cDNA clone MGC:71335 IMAGE:6088873	BC067086.1	-0.825	0.009	-1.25	2.37
<i>FLJ43880^c</i>	cDNA FLJ43880 fis, clone TEST14009022	AK125868.1	-0.796	0.017	-1.24	2.36
<i>ADAM33</i>	ADAM metallopeptidase domain 33	NM_153202.1	-0.709	0.027	-1.22	2.33
<i>RC6-HT0840-150800-022-D11</i> <i>HT0840</i>	RC6-HT0840-150800-022-D11 HT0840 Homo sapiens cDNA	BE719128.1	-0.678	0.031	-1.22	2.33
<i>FXD1</i>	FXD domain containing ion transport regulator 1 (phospholemman)	NM_021902.2	-0.578	0.044	-1.20	2.29
<i>MAF</i>	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	NM_005360.2	-0.568	0.044	-1.19	2.29

Continued

TABLE 2—Continued

<i>ABI3BP^c</i>	Abi gene family, member 3 (NESH) binding protein	NM_015429.2	−0.705	0.012	−1.17	2.26
<i>UI-E-DW1-ahc-b-11-0-Ulr1 UI-E-DW1^c</i>	UI-E-DW1-ahc-b-11-0-Ulr1 cDNA clone UI-E-DW1-ahc-b-11-0-UI 5'	BM712072.1	−0.854	0.008	−1.14	2.21
<i>MTSS1^b</i>	metastasis suppressor 1	NM_014751.2	−0.731	0.009	−1.14	2.20
<i>CLDN15^b</i>	claudin 15	NM_014343.1	−0.714	0.022	−1.13	2.19
<i>GANAB</i>	glucosidase, alpha; neutral AB	NM_198334.1	−0.719	0.020	−1.13	2.19
<i>C1QTNF1^c</i>	C1q and tumor necrosis factor related protein 1	NM_030968.2	−0.672	0.017	−1.13	2.18
<i>ATF3</i>	activating transcription factor 3	NM_004024.2	−0.619	0.049	−1.10	2.14
<i>C17orf58^c</i>	chromosome 17 open reading frame 58	NM_181655.1	−0.813	0.007	−1.10	2.14
<i>P2RY6^c</i>	pyrimidinergic receptor P2Y, G-protein coupled, 6	NM_004154.3	−0.814	0.021	−1.08	2.12
<i>ADAM8^c</i>	α disintegrin and metalloproteinase domain 8	NM_001109.1	−0.628	0.044	−1.06	2.08
<i>PNRC1</i>	proline-rich nuclear receptor coactivator 1	NM_006813.1	−0.902	0.008	−1.05	2.07
<i>FLJ23438^b</i>	cDNA: FLJ23438 fis, clone HRC13275	AK027091.1	−0.738	0.016	−1.04	2.06
<i>BTN3A3^c</i>	butyrophilin, subfamily 3, member A3	NM_197974.1	−0.833	0.009	−1.03	2.05
<i>UI-H-EU0-azp-α-24-0-Uls1 NCL_CGAP_Car1^b</i>	UI-H-EU0-azp-α-24-0-Uls1 NCI_CGAP_Car1 cDNA clone IMAGE: 5851679 3'	BQ181011.1	−0.716	0.009	−1.01	2.01
<i>CREB5^b</i>	cAMP responsive element binding protein 5	NM_004904.1	−0.9	0.010	−1.00	2.00
<i>HLA-G</i>	HLA-G histocompatibility antigen, class I, G	NM_002127.3	−0.721	0.028	−0.99	1.99
<i>MATN2</i>	matrilin 2	NM_030583.1	−0.743	0.027	−0.96	1.95
<i>HHLA2</i>	HERV-H LTR-associating 2	NM_007072.2	−0.488	0.024	−0.95	1.93
<i>HLA-F</i>	major histocompatibility complex, class I, F	NM_018950.1	−0.619	0.039	−0.95	1.93
<i>TXNIP</i>	thioredoxin interacting protein	NM_006472.1	−0.604	0.034	−0.94	1.92
<i>KLF11</i>	Kruppel-like factor 11	NM_003597.4	−0.493	0.039	−0.92	1.89
<i>ARID5A</i>	AT rich interactive domain 5A (MRF1-like)	NM_006673.2	−0.526	0.042	−0.92	1.89
<i>PERP</i>	PERP, TP53 apoptosis effector	NM_022121.2	−0.602	0.042	−0.92	1.89
<i>HHLA1</i>	HERV-H LTR-associating 1	NM_005712.1	−0.661	0.031	−0.92	1.89
<i>GYPC^b</i>	glycophorin C (Gerbich blood group)	NM_002101.3	−0.578	0.046	−0.91	1.88
<i>NUCB1</i>	nucleobindin 1	NM_006184.3	−0.618	0.026	−0.89	1.86
<i>LGALS3BP^b</i>	lectin, galactoside-binding, soluble, 3 binding protein	NM_005567.2	−0.784	0.014	−0.89	1.85
<i>FLJ20099^b</i>	cDNA FLJ20099 fis, clone COL04544	AK000106.1	−0.711	0.023	−0.89	1.85
<i>TMEM142C^b</i>	transmembrane protein 142C	NM_152288.1	−0.803	0.011	−0.87	1.83
<i>ATXN2L</i>	ataxin 2-like	NM_007245.2	−0.596	0.046	−0.87	1.83
<i>PAQR6^b</i>	progesterone and adipoQ receptor family member VI	NM_024897.2	−0.63	0.040	−0.87	1.83
<i>SPTBN1</i>	spectrin, beta, non-erythrocytic 1 (SPTBN1)	NM_003128.1	−0.62	0.049	−0.87	1.83
<i>MGC15875^b</i>	hypothetical protein MGC15875 (MGC15875)	NM_032921.1	−0.894	0.013	−0.86	1.81
<i>MAPK8IP1</i>	mitogen-activated protein kinase 8 interacting protein 1	NM_005456.2	−0.642	0.037	−0.86	1.81

Continued

TABLE 2—Continued

<i>ANKZF1</i>	ankyrin repeat and zinc finger domain containing 1	NM_018089.1	−0.673	0.036	−0.85	1.81
<i>CCBE1</i>	collagen and calcium binding EGF domains 1	NM_133459.1	−0.602	0.034	−0.85	1.81
<i>DKFZp779O1626</i>	mRNA; cDNA DKFZp779O1626 (from clone DKFZp779O1626)	BX648538.1	−0.676	0.027	−0.83	1.78
<i>CA12</i>	carbonic anhydrase XII	AK000158.1	−0.612	0.040	−0.83	1.78
<i>NFIL3^b</i>	nuclear factor, interleukin 3 regulated	NM_005384.1	−0.803	0.012	−0.83	1.77
<i>IFITM3</i>	interferon induced transmembrane protein 3 (1-8U)	NM_021034.1	−0.604	0.033	−0.83	1.77
<i>SLC27A1</i>	solute carrier family 27 (fatty acid transporter), member 1	NM_198580.1	−0.621	0.047	−0.81	1.75
<i>LAMP1</i>	lysosomal-associated membrane protein 1	NM_005561.2	−0.648	0.037	−0.80	1.74
<i>BCL3</i>	B-cell CLL/lymphoma 3	NM_005178.2	−0.671	0.039	−0.79	1.73
<i>R15896 ya47b07.r1</i>	R15896 ya47b07.r1 Soares infant brain 1NIB cDNA clone IMAGE:53125 5' similar to SP:S37009 S37009 TRANSPOSASE - ALMOND ;	R15896.1	−0.645	0.037	−0.79	1.73
<i>ZFP64^b</i>	zinc finger protein 64 homolog (mouse)	NM_018197.2	−0.795	0.017	−0.78	1.72
<i>SWAP-70^b</i>	SWAP-70 protein	AB014540.1	−0.708	0.028	−0.77	1.71
<i>P2RX4</i>	purinergic receptor P2X, ligand-gated ion channel, 4	NM_002560.2	−0.69	0.032	−0.77	1.71
<i>BMP1</i>	bone morphogenetic protein 1 (BMP1)	NM_006129.2	−0.598	0.048	−0.76	1.69
<i>SLC25A37</i>	solute carrier family 25, member 37	NM_016612.1	−0.607	0.039	−0.75	1.68
<i>NUB1^b</i>	negative regulator of ubiquitin-like proteins 1	NM_016118.3	−0.68	0.011	−0.75	1.68
<i>ACADS</i>	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	NM_000017.1	−0.669	0.046	−0.74	1.67
<i>RELB</i>	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	NM_006509.2	−0.682	0.033	−0.74	1.67
<i>PPARA^b</i>	peroxisome proliferative activated receptor, α	NM_032644.1	−0.753	0.009	−0.74	1.67
<i>ELL3^b</i>	elongation factor RNA polymerase II-like 3	NM_025165.1	−0.791	0.006	−0.74	1.67
<i>BCL6^c</i>	B-cell CLL/lymphoma 6 (zinc finger protein 51)	NM_001706.2	−0.819	0.013	−0.74	1.67
<i>PYGL^c</i>	phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	NM_002863.2	−0.804	0.016	−0.74	1.67
<i>cDNA clone fs01a03 5'</i>	fs01a03y1 Human Lens cDNA (Normalized): fs cDNA clone fs01a03 5'	CD673362.1	−0.615	0.030	−0.73	1.66
<i>602156641F1 NIH_MGC_83</i>	602156641F1 NIH_MGC_83 cDNA clone IMAGE:4297258 5'	BF681513.1	−0.597	0.037	−0.73	1.66
<i>CSNK2A1</i>	casein kinase 2, α 1 polypeptide	NM_177560.2	−0.693	0.030	−0.73	1.66
<i>KIAA0082</i>	KIAA0082	NM_015050.1	−0.945	0.010	−0.72	1.65
<i>UI-E-DW0-agh-c-03-0-UIr1 UI-E-DW0^c</i>	UI-E-DW0-agh-c-03-0-UIr1 UI-E-DW0 cDNA clone UI-E-DW0-agh-c-03-0-UI 5'	BM706230.1	−0.714	0.024	−0.72	1.65
<i>CNTNAP1</i>	contactin associated protein 1	NM_003632.1	−0.628	0.041	−0.72	1.64

Continued

TABLE 2—Continued

<i>SYNGR1^b</i>	synaptogyrin 1	NM_004711.3	−0.635	0.043	−0.71	1.64
<i>ING4</i>	inhibitor of growth family, member 4	NM_198287.1	−0.586	0.044	−0.69	1.62
<i>RBM5^b</i>	RNA binding motif protein 5	NM_005778.1	−0.71	0.024	−0.69	1.61
<i>FLJ34274</i>	cDNA FLJ34274 fis, clone FEBRA2003327	AK091593.1	−0.618	0.044	−0.68	1.60
<i>FAM113A^b</i>	family with sequence similarity 113, member A	NM_022760.3	−0.796	0.015	−0.68	1.60
<i>MAP3K6</i>	mitogen-activated protein kinase kinase kinase 6	NM_004672.3	−0.584	0.041	−0.67	1.59
<i>FLJ14360</i>	hypothetical protein FLJ14360	NM_032775.2	−0.638	0.030	−0.67	1.59
<i>B4GALT7^b</i>	xylosylprotein β 1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase I)	NM_007255.1	−0.76	0.026	−0.66	1.57
<i>CASP7^b</i>	caspase 7, apoptosis-related cysteine peptidase	NM_001227.2	−0.637	0.023	−0.65	1.57
<i>IXL^b</i>	intersex-like (Drosophila)	AL137304.1	−0.662	0.031	−0.65	1.57
<i>RHBDF2</i>	rhomboid-like protein 6	NM_024599.2	−0.636	0.030	−0.65	1.57
<i>UCN</i>	urocortin	NM_003353.2	−0.625	0.044	−0.65	1.57
<i>FLRT2</i>	fibronectin leucine rich transmembrane protein 2	NM_013231.3	−0.595	0.042	−0.64	1.56
<i>FLJ45129</i>	cDNA FLJ45129 fis, clone BRAWH3037394	AK127072.1	−0.582	0.048	−0.63	1.55
<i>LKAP^c</i>	limkain b1	NM_014647.1	−0.693	0.012	−0.63	1.55
<i>yj34b04r1^b</i>	yj34b04r1 Soares placenta Nb2HP cDNA clone IMAGE:150607 5'	H02050.1	−0.722	0.017	−0.62	1.54
<i>HECA^b</i>	headcase homolog (Drosophila)	NM_016217.1	−0.629	0.047	−0.62	1.54
<i>BMP1^b</i>	bone morphogenetic protein 1	NM_006129.2	−0.763	0.024	−0.62	1.54
<i>601194047F1 NIH_MGC_7</i>	601194047F1 NIH_MGC_7 cDNA clone IMAGE:3537995 5'	BE264943.1	−0.699	0.048	−0.62	1.54
<i>TNIP2^b</i>	TNFAIP3 interacting protein 2	NM_024309.2	−0.95	0.008	−0.62	1.54
<i>ZNF688</i>	zinc finger protein 688	NM_145271.2	−0.669	0.026	−0.61	1.53
<i>MITF</i>	microphthalmia-associated transcription factor	NM_006722.1	−0.62	0.042	−0.60	1.51
<i>FLJ45204^b</i>	cDNA FLJ45204 fis, clone BRCAN2009168	AK127147.1	−0.807	0.010	−0.60	1.51
<i>UBXD7^c</i>	UBX domain containing 7	AB018337.1	−0.811	0.007	−0.58	1.50
<i>C16orf48^b</i>	hypothetical protein DKFZp434A1319 (DKFZP434A1319)	NM_032140.1	−0.714	0.015	−0.58	1.50
<i>ZFX4</i>	zinc finger homeodomain 4	NM_024721.2	−0.643	0.030	−0.58	1.49
<i>yo60a08r1</i>	yo60a08r1 Soares breast 3NbHBst cDNA clone IMAGE:182294 5' similar to contains Alu repetitive element; contains LTR9 repetitive element	H41942.1	−0.692	0.031	−0.58	1.49
<i>ZNF44</i>	zinc finger protein 44	BC032246.1	−0.614	0.032	−0.57	1.49
<i>SETD5</i>	SET domain containing 5	AB051544.1	−0.6	0.031	−0.57	1.48
<i>RNF103</i>	ring finger protein 103	NM_005667.2	−0.648	0.031	−0.57	1.48
<i>TP53INP1</i>	tumor protein p53 inducible nuclear protein 1	NM_033285.2	−0.642	0.028	−0.55	1.47
<i>yr35b01r1 Soares fetal liver spleen 1NFLS^b</i>	yr35b01r1 Soares fetal liver spleen 1NFLS cDNA clone IMAGE:207241 5'	H59642.1	−0.631	0.038	−0.55	1.46
<i>BBS1</i>	Bardet-Biedl syndrome 1	NM_024649.4	−0.616	0.047	−0.54	1.46
<i>FLJ43900</i>	cDNA FLJ43900 fis, clone TEST14009973	AK125888.1	−0.669	0.043	−0.54	1.45
<i>ITM2B^b</i>	integral membrane protein 2B	NM_021999.2	−0.681	0.028	−0.54	1.45
<i>PDE6G^b</i>	phosphodiesterase 6G, cGMP-specific, rod, gamma	NM_002602.1	−0.87	0.003	−0.53	1.44

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TABLE 2—Continued

<i>FKBP4</i>	FK506 binding protein 4, 59kDa	NM_002014.2	−0.697	0.023	−0.53	1.44
<i>CHKB</i>	choline kinase β (CHKB)	NM_005198.3	−0.593	0.039	−0.52	1.43
<i>FLJ11946^b</i>	cDNA FLJ11946 fis, clone HEMBB1000709	AK022008.1	−0.922	0.005	−0.50	1.42
<i>wi65f08x1</i>	wi65f08x1 NCI_CGAP_Kid12 cDNA	AI763182.1	−0.621	0.044	−0.50	1.42
<i>NCL</i>	clone IMAGE:2398215 3'					
<i>CGAP_Kid12</i>						
<i>NSMAF^c</i>	neutral sphingomyelinase (N-SMase) activation associated factor	NM_003580.2	−0.789	0.015	−0.50	1.41
<i>SIX5^b</i>	sine oculis homeobox homolog 5 (Drosophila)	NM_175875.3	−0.75	0.017	−0.49	1.40
<i>64B2 Human retina cDNA Tsp509I-cleaved sublibrary^b</i>	64B2 Human retina cDNA Tsp509I-cleaved sublibrary Homo sapiens cDNA not directional	W22248.1	−0.684	0.022	−0.48	1.39
<i>XPC</i>	xeroderma pigmentosum, complementation group C	NM_004628.2	−0.644	0.041	−0.48	1.39
<i>603040523F1 NIH_MGC_115</i>	603040523F1 NIH_MGC_115 cDNA clone IMAGE:5181509 5'	BI824163.1	−0.708	0.030	−0.48	1.39
<i>DKFZp686K2137</i>	mRNA; cDNA DKFZp686K2137 (from clone DKFZp686K2137)	AL833529.1	−0.626	0.037	−0.47	1.38
<i>SRRM2</i>	serine/arginine repetitive matrix 2	NM_016333.2	−0.632	0.042	−0.45	1.36
<i>SH3GLB2</i>	SH3-domain GRB2-like endophilin B2	NM_020145.2	−0.662	0.037	−0.45	1.36
<i>yd89d12r1 Soares fetal liver spleen 1NFLS</i>	yd89d12r1 Soares fetal liver spleen 1NFLS cDNA clone IMAGE:115415 5'	T87528.1	−0.592	0.050	−0.43	1.35
<i>cDNA clone IMAGE:3636922</i>	cDNA clone IMAGE:3636922	BC062348.1	−0.598	0.035	−0.43	1.34
<i>PTPRA</i>	protein tyrosine phosphatase, receptor type, A	NM_002836.2	−0.624	0.042	−0.41	1.33
<i>C20orf111</i>	chromosome 20 open reading frame 111	NM_016470.6	−0.632	0.043	−0.41	1.33
<i>MGAT1^b</i>	mannosyl (α -1,3-)-glycoprotein β -1,2-N-acetylglucosaminyltransferase	NM_002406.2	−0.799	0.019	−0.40	1.32
<i>ZDHHC3^b</i>	zinc finger, DHHC domain containing 3	NM_016598.1	−0.634	0.040	−0.32	1.25
<i>cDNA DKFZp313K127^b</i>	mRNA; cDNA DKFZp313K127 (from clone DKFZp313K127)	AL833316.1	−0.74	0.024	−0.31	1.24
<i>GANAB</i>	glucosidase, alpha; neutral AB	NM_198334.1	−0.676	0.034	−0.31	1.24
					x-fold up-regulated in Apert	
<i>NUBP2</i>	nucleotide binding protein 2 (MinD homolog, E coli)	NM_012225.1	0.66	0.030	0.23	1.17
<i>AGENCOURT_8454944 NIH_MGC_113</i>	AGENCOURT_8454944 NIH_MGC_113 cDNA clone IMAGE:6278846 5'	BU899205.1	0.587	0.046	0.28	1.22
<i>ATP5G1</i>	ATP synthase, H + transporting, mitochondrial F0 complex, subunit C1 (subunit 9)	NM_005175.1	0.618	0.042	0.28	1.22

Continued

TABLE 2—Continued

AGENCOURT_8073800 NIH_MGC_110 ^b	AGENCOURT_8073800 NIH_MGC_110 cDNA clone IMAGE:6083397 5'	BU147450.1	0.678	0.020	0.32	1.25
CIB1 ^b	calcium and integrin binding 1 (calmyrin)	NM_006384.2	0.665	0.035	0.32	1.25
ARL3	ADP-ribosylation factor-like 3	NM_004311.2	0.633	0.033	0.34	1.27
PSMA2	proteasome (prosome, macropain) subunit, α type, 2	NM_002787.3	0.648	0.039	0.36	1.28
MAGED2 ^b	melanoma antigen, family D, 2	NM_014599.4	0.599	0.030	0.36	1.28
CUTA	cutA divalent cation tolerance homolog (E. coli)	NM_015921.1	0.603	0.044	0.37	1.29
CSNK1D	casein kinase 1, delta	NM_001893.3	0.614	0.042	0.37	1.29
MTCH2	mitochondrial carrier homolog 2 (C elegans) (MTCH2), nuclear gene encoding mitochondrial protein	NM_014342.2	0.576	0.045	0.38	1.30
yp83b01s1 Soares fetal liver spleen 1NFLS	yp83b01s1 Soares fetal liver spleen 1NFLS cDNA clone IMAGE:193993 3'	H51256.1	0.597	0.049	0.38	1.30
MAPRE1	microtubule-associated protein, RP/EB family, member 1	NM_012325.1	0.613	0.045	0.38	1.30
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	NM_003072.2	0.658	0.034	0.39	1.31
PLA2G12A	phospholipase A2, group X1IA	NM_030821.3	0.668	0.044	0.39	1.31
METTL2B ^b	methyltransferase like 2	NM_018396.1	0.753	0.019	0.40	1.32
EEF1B2 ^c	eukaryotic translation elongation factor 1 β 2	NM_001959.2	0.879	0.011	0.40	1.32
GABPB2	GA binding protein transcription factor, β subunit 2	NM_002041.2	0.664	0.027	0.40	1.32
ZNF414 ^b	zinc finger protein 414	NM_032370.1	0.742	0.029	0.41	1.32
ADCY2 ^b	adenylate cyclase 2 (brain)	NM_020546.1	0.665	0.028	0.41	1.33
CASP8AP2	CASP8 associated protein 2	NM_012115.2	0.621	0.049	0.41	1.33
TIMP3 ^c	TIMP metalloproteinase inhibitor 3	BG104808.1	0.966	0.006	0.42	1.34
EIF4A1	eukaryotic translation initiation factor 4A	NM_001416.1	0.585	0.038	0.44	1.35
MUS81 ^b	MUS81 endonuclease homolog (S. cerevisiae)	NM_025128.3	0.662	0.024	0.44	1.35
CCDC72	coiled-coil domain containing 72	NM_015933.1	0.694	0.030	0.44	1.36
SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	NM_006938.2	0.619	0.044	0.44	1.36
DTYMK	deoxythymidylate kinase	NM_012145.2	0.64	0.037	0.44	1.36
STX2 ^b	syntaxin 2	NM_194356.1	0.745	0.019	0.44	1.36
AARSD1 ^b	alanyl-tRNA synthetase domain containing 1	NM_025267.2	0.786	0.019	0.45	1.37
CREB3 ^b	cAMP responsive element binding protein 3	NM_006368.4	0.743	0.023	0.46	1.38
COX4NB	neighbor of COX4 (NOC4)	NM_006067.3	0.593	0.030	0.47	1.38
PDZK6	PDZ domain containing 6	NM_015693.2	0.63	0.038	0.47	1.39
UBE2N	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	NM_003348.3	0.584	0.037	0.48	1.39
PPIH	peptidyl prolyl isomerase H	NM_006347.3	0.676	0.025	0.48	1.40
SIKE	suppressor of IKK epsilon	NM_025073.1	0.584	0.048	0.50	1.41
TK1	thymidine kinase 1, soluble	NM_003258.1	0.685	0.028	0.50	1.41

Continued

TABLE 2—Continued

<i>PAFAH1B3</i>	platelet-activating factor acetylhydrolase, isoform Ib, γ subunit 29kDa	NM_002573.2	0.651	0.037	0.51	1.42
<i>SFRS7^b</i>	splicing factor, arginine/serine-rich 7, 35kDa	NM_006276.36	0.705	0.028	0.52	1.44
<i>PSMC5</i>	proteasome (prosome, macropain) 26S subunit, ATPase, 5	NM_002805.4	0.624	0.043	0.53	1.44
<i>yr11c06r1</i> Soares fetal liver spleen 1NFLS ^b	yr11c06r1 Soares fetal liver spleen 1NFLS cDNA clone IMAGE:204970 5'	H57392.1	0.742	0.015	0.53	1.44
<i>C15orf29^b</i>	chromosome 15 open reading frame 29	NM_024713.1	0.793	0.011	0.53	1.44
<i>GFOD2^c</i>	glucose-fructose oxidoreductase domain containing 2	NM_030819.2	0.745	0.019	0.53	1.45
<i>HN1</i>	hematological and neurological expressed 1	NM_001002032.1	0.683	0.045	0.54	1.46
<i>DKFZp451H129</i>	mRNA; cDNA DKFZp451H129 (from clone DKFZp451H129)	AL833295.1	0.649	0.035	0.54	1.46
<i>CKLF^b</i>	chemokine-like factor	NM_016951.2	0.726	0.021	0.55	1.46
<i>OBFC1</i>	oligonucleotide/oligosaccharide- binding fold containing 1	NM_024928.3	0.688	0.027	0.55	1.46
<i>VAMP4^b</i>	vesicle-associated membrane protein 4, transcript variant 1	NM_201994.1	0.744	0.010	0.55	1.46
<i>RRM1</i>	ribonucleotide reductase M1 polypeptide	NM_001033.2	0.612	0.045	0.55	1.46
<i>PSME4^b</i>	proteasome (prosome, macropain) activator subunit 4	NM_014614.1	0.736	0.022	0.56	1.47
<i>PLK3</i>	polo-like kinase 3 (Drosophila)	NM_004073.2	0.642	0.036	0.57	1.48
<i>MRPL35^b</i>	mitochondrial ribosomal protein L35	NM_016622.2	0.706	0.014	0.57	1.49
<i>DLAT^b</i>	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	NM_001931.2	0.707	0.019	0.58	1.49
<i>HIP2^b</i>	huntingtin interacting protein 2	NM_005339.3	0.723	0.017	0.58	1.49
<i>C20orf7</i>	chromosome 20 open reading frame 7	NM_199052.1	0.615	0.041	0.59	1.50
<i>UFD1L^b</i>	ubiquitin fusion degradation 1 like (yeast)	NM_005659.3	0.616	0.023	0.59	1.51
<i>POP5</i>	processing of precursor 5, ribonuclease P/MRP subunit (S cerevisiae) (POP5)	NM_015918.3	0.623	0.041	0.59	1.51
<i>SLC35B3</i>	solute carrier family 35, member B3	NM_015948.2	0.628	0.037	0.60	1.51
<i>CDKN3</i>	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	NM_005192.2	0.613	0.044	0.60	1.52
<i>LZIC^b</i>	leucine zipper and CTNNBIP1 domain containing	NM_032368.3	0.766	0.019	0.60	1.52
<i>LETM1</i>	leucine zipper-EF-hand containing transmembrane protein 1	NM_012318.1	0.589	0.049	0.60	1.52
<i>RNASEH2A^b</i>	ribonuclease H2, subunit A	NM_006397.2	0.869	0.011	0.60	1.52
<i>NEDD1</i>	neural precursor cell expressed, developmentally downregulated 1	NM_152905.2	0.643	0.041	0.62	1.53
<i>CEP78</i>	centrosomal protein 78kDa	AW962072.1	0.672	0.028	0.63	1.54
<i>UI-H-B10-aah- e-06-0-Uls1</i> NCL_ CGAP_ Sub1	UI-H-B10-aah-e-06-0-Uls1 NCL_CGAP_ Sub1 cDNA clone IMAGE:2709227 3'	AW014009.1	0.597	0.037	0.63	1.55
<i>CKAP2</i>	cytoskeleton associated protein 2	NM_018204.2	0.644	0.037	0.66	1.58

Continued

TABLE 2—Continued

<i>ACTR3B^b</i>	ARP3 actin-related protein 3 homolog B (yeast)	NM_020445.1	0.765	0.016	0.67	1.59
<i>ELAVL1</i>	ELAV (embryonic lethal, abnormal vision, <i>Drosophila</i>)-like 1 (Hu antigen R)	NM_001419.2	0.635	0.041	0.67	1.59
<i>POLR3K^b</i>	polymerase (RNA) III (DNA directed) polypeptide K	NM_016310.2	0.802	0.013	0.69	1.61
<i>PI4KI^b</i>	phosphatidylinositol 4-kinase type II	NM_018425.2	0.77	0.014	0.69	1.62
<i>TMEM107^b</i>	transmembrane protein 107	NM_183065.1	0.739	0.022	0.70	1.62
<i>BCAP29</i>	B-cell receptor-associated protein 29	NM_018844.1	0.644	0.040	0.70	1.63
<i>PRPS1L1^b</i>	phosphoribosyl pyrophosphate synthetase 1-like 1	NM_175886.2	0.728	0.022	0.71	1.63
<i>MTFMT^b</i>	mitochondrial methionyl-tRNA formyltransferase	NM_139242.2	0.866	0.008	0.72	1.64
<i>DDX49</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 49	NM_019070.3	0.61	0.046	0.73	1.66
<i>RAD1^b</i>	RAD1 homolog (<i>S. pombe</i>)	NM_002853.2	0.71	0.018	0.73	1.66
<i>CMTM5</i>	CKLF-like MARVEL transmembrane domain containing 5	NM_138460.2	0.721	0.019	0.74	1.67
<i>NEK6^b</i>	NIMA (never in mitosis gene a)-related kinase 6	NM_014397.3	0.715	0.017	0.74	1.67
<i>K-EST0149992</i> <i>L3SNU475^b</i>	K-EST0149992 L3SNU475 cDNA clone L3SNU475-29-H12 5'	CB108944.1	0.657	0.015	0.75	1.68
<i>yx15f01s1</i> <i>Soares</i> <i>melanocyte</i> <i>2NbHM^b</i>	yx15f01s1 Soares melanocyte 2NbHM cDNA clone IMAGE:261817 3'	H99198.1	0.766	0.015	0.76	1.70
<i>EXOSC9^b</i>	exosome component 9	NM_005033.1	0.721	0.021	0.76	1.70
<i>UI-E-C10-aah-e-03-0-Ulr1</i> <i>UI-E-C10</i>	UI-E-C10-aah-e-03-0-Ulr1 UI-E-C10 cDNA clone UI-E-C10-aah-e-03-0-UI 5'	BM690376.1	0.659	0.031	0.79	1.73
<i>PTS</i>	6-pyruvoyltetrahydropterin synthase	NM_000317.1	0.597	0.047	0.79	1.73
<i>C14orf143</i>	chromosome 14 open reading frame 143	NM_145231.1	0.629	0.047	0.79	1.73
<i>UBE2T</i>	HSPC150 protein similar to ubiquitin-conjugating enzyme	NM_014176.1	0.664	0.029	0.83	1.77
<i>SAE1</i>	SUMO-1 activating enzyme subunit 1	NM_005500.1	0.62	0.049	0.83	1.78
<i>AURKB</i>	aurora kinase B	NM_004217.1	0.692	0.031	0.84	1.79
<i>ARSJ</i>	arylsulfatase family, member J	AK027201.1	0.708	0.028	0.85	1.80
<i>RRM2^b</i>	ribonucleotide reductase M2 polypeptide	NM_001034.1	0.781	0.019	0.86	1.81
<i>BCL7A</i>	B-cell CLL/lymphoma 7A	NM_020993.2	0.652	0.034	0.86	1.82
<i>IVNS1ABP</i>	influenza virus NS1A binding protein	NM_006469.2	0.66	0.035	0.87	1.82
<i>NCAPG</i>	non-SMC condensin I complex, subunit G	NM_022346.2	0.689	0.028	0.87	1.83
<i>CENPM^c</i>	centromere protein M	NM_024053.2	0.693	0.006	0.87	1.83
<i>C5orf13</i>	chromosome 5 open reading frame 13	NM_004772.1	0.626	0.042	0.89	1.85
<i>MCM4</i>	MCM4 minichromosome maintenance deficient 4 (<i>S. cerevisiae</i>)	NM_182746.1	0.65	0.030	0.90	1.87
<i>FLJ43294</i>	cDNA FLJ43294 fis, clone MESTC1000042	AK125284.1	0.527	0.049	0.93	1.90
<i>PTPDC1^b</i>	protein tyrosine phosphatase domain containing 1	NM_152422.3	0.725	0.027	0.95	1.93
<i>LOC90624</i>	hypothetical protein LOC90624	NM_181705.1	0.642	0.026	0.95	1.94
<i>SPAG5^b</i>	sperm associated antigen 5	NM_006461.2	0.876	0.007	0.98	1.97
<i>CTPS</i>	CTP synthase	NM_001905.1	0.496	0.046	0.98	1.98
<i>zu70f09r1</i> <i>Soares_</i> <i>testis_NHT</i>	zu70f09r1 Soares_testis_NHT cDNA clone IMAGE:743369 5'	AA400694.1	0.578	0.039	0.99	1.98

Continued

TABLE 2—Continued

<i>MND1</i>	meiotic nuclear divisions 1 homolog (S. cerevisiae)	NM_032117.2	0.674	0.034	1.00	2.00
<i>KIAA0101</i>	KIAA0101 gene product	NM_014736.3	0.58	0.050	1.02	2.03
<i>ZNF738^c</i>	zinc finger protein 738	BC034499.1	1.07	0.003	1.03	2.04
<i>EHD4^c</i>	EH-domain containing 4	NM_139265.2	0.725	0.001	1.04	2.06
<i>MNS1^b</i>	meiosis-specific nuclear structural 1	NM_018365.1	0.832	0.013	1.06	2.08
<i>ARHGAP11A</i>	similar to human GTPase-activating protein	NM_014783.2	0.7	0.027	1.08	2.12
<i>CENPN^c</i>	centromere protein N	NM_018455.3	1.142	0.001	1.10	2.14
<i>STMN1^b</i>	stathmin 1/oncoprotein 18	NM_203401.1	0.969	0.006	1.11	2.16
<i>DUSP2^b</i>	dual specificity phosphatase 2	NM_004418.2	0.953	0.004	1.17	2.25
<i>SPBC25^b</i>	spindle pole body component 25 homolog (S. cerevisiae)	NM_020675.3	0.892	0.010	1.18	2.27
<i>HIST1H4C</i>	histone 1, H4c	NM_003542.3	0.682	0.033	1.22	2.32
<i>TCF19</i>	transcription factor 19 (SC1)	BC033086.1	0.631	0.041	1.23	2.34
<i>CIP29</i>	cytokine induced protein 29 kDa	NM_033082.1	0.65	0.040	1.24	2.36
<i>FLJ39342^b</i>	cDNA FLJ39342 fis, clone OCBBF2018873	AK096661.1	0.776	0.017	1.32	2.50
<i>FLJ23131</i>	cDNA: FLJ23131 fis, clone LNG08502	AK026784.1	0.645	0.039	1.61	3.05
<i>zv03f03r1</i>	zv03f03r1 Soares_NhHMPu_S1 cDNA	AA419568.1	0.718	0.041	1.74	3.34
Soares_NhHMPu_S1	clone IMAGE:752573 5'					
<i>CNN1</i>	calponin 1, basic, smooth muscle	NM_001299.3	0.808	0.022	2.09	4.24
<i>HAPLN1^c</i>	hyaluronan and proteoglycan link protein 1	NM_001884.2	1.003	0.005	3.00	7.99

^aSNR stands for "signal to noise ratio".

^bThe 120 genes identified as differentially expressed in at least 50% of the "leave-one-out" datasets.

^cThe 25 genes identified as differentially expressed in 100% of the "leave-one-out" datasets.

To improve the statistical significance and identify the most robust markers in the AS gene expression signature we performed a leave-one-out statistical analysis (detailed in Methods). Out of the 263 genes previously found to be differentially expressed, 120 genes were identified in at least 50 percent of the "leave-one-out" datasets, and 25 genes were present in all "leave-one-out" datasets (Tables 2 and 3).

Quantitative Real Time PCR Validation of Microarray Data

Quantitative real-time PCR (qRT-PCR) was performed to confirm the differential expression of gene sets identified in the microarray analysis. We selected for validation four genes belonging to the previously mentioned functional categories: *STMN1* and *SPAG5* (cell proliferation), *RRM2* (cell proliferation and nucleotide metabolism), and *HIP2* (suppression of apoptosis and regulation

of gene expression). In addition, two other genes were selected with non-related functions: *CENPN* (centromere protein), and *EEF1B2* (translation elongation factor). The above selection was picked at random to avoid a bias toward a specific pathway in the validation of the microarray results. qRT-PCR experiments showed that these six genes were upregulated in AS cells (Figure 3), corroborating the expression results obtained in the microarray experiments.

FGF2 Treatment

To independently confirm the data generated by microarray analysis, and to mimic the increased downstream signaling caused by mutant FGFR2, primary cultured control periosteal cells were treated with a high concentration of FGF2 (the high concentration of FGF2 was used to simulate the phosphorylation status of mutant FGFR2c). Total RNA was isolated three, six, and 24 h

after FGF2 addition. qRT-PCR was used to measure the changes in mRNA levels of the six genes previously used for experimental validation of the microarray data: *STMN1*, *SPAG5*, *RRM2*, *HIP2*, *CENPN* and *EEF1B2*. We observed that FGF2 treatment differentially stimulated the expression of these genes only after 24 h of treatment (Figure 4).

DISCUSSION

Despite the important role played by the periosteum in normal suture biology, the contribution of FGFR2 mutant periosteal cells in AS suture closure is unknown. We report here, for the first time, a strikingly higher osteoblast differentiation of heterozygous p.Ser252Trp FGFR2 mutant cells as compared with the same kind of cells from individuals without this mutation and normal suture fusion. To delineate molecular mechanisms underlying this abnormal cell behavior, we also present here a statistically significant difference in gene

Table 3. 98 genes grouped in functional categories. Transcripts are ordered by their Apert-to-control ratios (Log 2). Upregulated genes in AS cells are shown in orange. Downregulated genes are shown in blue.

Gene (Official Symbol)	Gene name	Accession number	P value	Apert-to- control Ratio (Log2)	x-fold up- or down- regulated in Apert
Positive regulation of cell proliferation					
<i>CIP29</i>	cytokine induced protein 29 kDa	NM_033082.1	0.040	1.24	2.36
<i>TCF19</i>	transcription factor 19 (SC1)	BC033086.1	0.041	1.23	2.34
<i>SPBC25^b</i>	spindle pole body component 25 homolog (<i>S. cerevisiae</i>)	NM_020675.3	0.010	1.18	2.27
<i>STMN1^b</i>	stathmin 1/oncoprotein 18	NM_203401.1	0.006	1.11	2.16
<i>SPAG5^b</i>	sperm associated antigen 5	NM_006461.2	0.007	0.98	1.97
<i>MCM4</i>	MCM4 minichromosome maintenance deficient 4 (<i>S. cerevisiae</i>)	NM_182746.1	0.030	0.90	1.87
<i>HCAP-G</i>	chromosome condensation protein G	NM_022346.2	0.028	0.87	1.83
<i>RRM2^b</i>	ribonucleotide reductase M2 polypeptide	NM_001034.1	0.019	0.86	1.81
<i>AURKB</i>	aurora kinase B	NM_004217.1	0.031	0.84	1.79
<i>NEK6^b</i>	NIMA (never in mitosis gene a)-related kinase 6	NM_014397.3	0.017	0.74	1.67
<i>CKAP2</i>	cytoskeleton associated protein 2	NM_018204.2	0.037	0.66	1.58
<i>NEDD1</i>	neural precursor cell expressed, developmentally downregulated 1	NM_152905.2	0.041	0.62	1.53
<i>RNASEH2A^b</i>	ribonuclease H2, subunit A	NM_006397.2	0.011	0.60	1.52
<i>UFD1L^b</i>	ubiquitin fusion degradation 1 like (yeast)	NM_005659.3	0.023	0.59	1.51
<i>PLK3</i>	polo-like kinase 3 (<i>Drosophila</i>)	NM_004073.2	0.036	0.57	1.48
<i>CKLF^b</i>	chemokine-like factor	NM_016951.2	0.021	0.55	1.46
<i>TK1</i>	thymidine kinase 1, soluble	NM_003258.1	0.028	0.50	1.41
<i>DTYMK</i>	deoxythymidylate kinase	NM_012145.2	0.037	0.44	1.36
<i>MUS81^b</i>	MUS81 endonuclease homolog (<i>S. cerevisiae</i>)	NM_025128.3	0.024	0.44	1.35
<i>MAPRE1</i>	microtubule-associated protein, RP/EB family, member 1	NM_012325.1	0.045	0.38	1.30
<i>HECA^b</i>	headcase homolog (<i>Drosophila</i>)	NM_016217.1	0.047	-0.62	1.54
<i>CSNK2A1</i>	casein kinase 2, α 1 polypeptide	NM_177560.2	0.030	-0.73	1.66
<i>BCL6^a</i>	B-cell CLL/lymphoma 6 (zinc finger protein 51)	NM_001706.2	0.013	-0.74	1.67
<i>BCL3</i>	B-cell CLL/lymphoma 3	NM_005178.2	0.039	-0.79	1.73
<i>ADAM33</i>	ADAM metalloproteinase domain 33	NM_153202.1	0.027	-1.22	2.33
<i>PDGFRA</i>	platelet-derived growth factor receptor, α polypeptide	NM_006206.2	0.045	-1.34	2.53
<i>ChGn^a</i>	chondroitin beta1,4 N-acetylgalactosaminyltransferase	NM_018371.3	0.015	-1.42	2.68
<i>PIK3C2B^a</i>	phosphoinositide-3-kinase, class 2, β polypeptide	NM_002646.2	0.004	-1.54	2.91
Negative regulation of cell proliferation					
<i>RAD1^b</i>	RAD1 homolog (<i>S. pombe</i>)	NM_002853.2	0.018	0.73	1.66
<i>CDKN3</i>	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	NM_005192.2	0.044	0.60	1.52
<i>RBM5^b</i>	RNA binding motif protein 5	NM_005778.1	0.024	-0.69	1.61
<i>ING4</i>	inhibitor of growth family, member 4	NM_198287.1	0.044	-0.69	1.62
<i>IFITM3</i>	interferon induced transmembrane protein 3 (1-8U)	NM_021034.1	0.033	-0.83	1.77
<i>KLF11</i>	Kruppel-like factor 11	NM_003597.4	0.039	-0.92	1.89
<i>MTSS1^b</i>	metastasis suppressor 1	NM_014751.2	0.009	-1.14	2.20
<i>RARRES3^b</i>	retinoic acid receptor responder (tazarotene induced) 3	NM_004585.2	0.022	-1.64	3.12
Nucleotide metabolism					
<i>CTPS</i>	CTP synthase	NM_001905.1	0.046	0.98	1.98
<i>RRM2^b</i>	ribonucleotide reductase M2 polypeptide	NM_001034.1	0.019	0.86	1.81
<i>PRPS1L1^b</i>	phosphoribosyl pyrophosphate synthetase 1-like 1	NM_175886.2	0.022	0.71	1.63
<i>POLR3K^b</i>	polymerase (RNA) III (DNA directed) polypeptide K	NM_016310.2	0.013	0.69	1.61
<i>RRM1</i>	ribonucleotide reductase M1 polypeptide	NM_001033.2	0.045	0.55	1.46
<i>TK1</i>	thymidine kinase 1, soluble	NM_003258.1	0.028	0.50	1.41
<i>DTYMK</i>	deoxythymidylate kinase	NM_012145.2	0.037	0.44	1.36

Continued

TABLE 3—Continued

<i>ADCY2^b</i>	adenylate cyclase 2 (brain)	NM_020546.1	0.028	0.41	1.33
<i>ATP5G1</i>	ATP synthase, H + transporting, mitochondrial F0 complex, subunit C1 (subunit 9)	NM_005175.1	0.042	0.28	1.22
<i>PDE6G^b</i>	phosphodiesterase 6G, cGMP-specific, rod, gamma	NM_002602.1	0.003	-0.53	1.44
Regulation of gene expression					
<i>CIP29</i>	cytokine induced protein 29 kDa	NM_033082.1	0.040	1.24	2.36
<i>ZNF738^a</i>	zinc finger protein 738	BC034499.1	0.003	1.03	2.04
<i>POLR3K^b</i>	polymerase (RNA) III (DNA directed) polypeptide K	NM_016310.2	0.013	0.69	1.61
<i>ELAVL1</i>	ELAV (embryonic lethal, abnormal vision, <i>Drosophila</i>)-like 1 (Hu antigen R)	NM_001419.2	0.041	0.67	1.59
<i>HIP2^b</i>	huntingtin interacting protein 2	NM_005339.3	0.017	0.58	1.49
<i>CREB3^b</i>	cAMP responsive element binding protein 3	NM_006368.4	0.023	0.46	1.38
<i>CASP8AP2</i>	CASP8 associated protein 2	NM_012115.2	0.049	0.41	1.33
<i>ZNF414^b</i>	zinc finger protein 414	NM_032370.1	0.029	0.41	1.32
<i>GABPB2</i>	GA binding protein transcription factor, β subunit 2	NM_002041.2	0.027	0.40	1.32
<i>SMARCA4</i>	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	NM_003072.2	0.034	0.39	1.31
<i>SIX5^b</i>	sine oculis homeobox homolog 5 (<i>Drosophila</i>)	NM_175875.3	0.017	-0.49	1.40
<i>ZNF44</i>	zinc finger protein 44	BC032246.1	0.032	-0.57	1.49
<i>ZFX4</i>	zinc finger homeodomain 4	NM_024721.2	0.030	-0.58	1.49
<i>MITF</i>	microphthalmia-associated transcription factor	NM_006722.1	0.042	-0.60	1.51
<i>ZNF688</i>	zinc finger protein 688	NM_145271.2	0.026	-0.61	1.53
<i>TNIP2^b</i>	TNFAIP3 interacting protein 2	NM_024309.2	0.008	-0.62	1.54
<i>ING4</i>	inhibitor of growth family, member 4	NM_198287.1	0.044	-0.69	1.62
<i>BCL6^a</i>	B-cell CLL/lymphoma 6 (zinc finger protein 51)	NM_001706.2	0.013	-0.74	1.67
<i>ELL3^b</i>	elongation factor RNA polymerase II-like 3	NM_025165.1	0.006	-0.74	1.67
<i>PPARA^b</i>	peroxisome proliferative activated receptor, α	NM_032644.1	0.009	-0.74	1.67
<i>RELB</i>	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	NM_006509.2	0.033	-0.74	1.67
<i>ZFP64^b</i>	zinc finger protein 64 homolog (mouse)	NM_018197.2	0.017	-0.78	1.72
<i>BCL3</i>	B-cell CLL/lymphoma 3	NM_005178.2	0.039	-0.79	1.73
<i>NFIL3^b</i>	nuclear factor, interleukin 3 regulated	NM_005384.1	0.012	-0.83	1.77
<i>ANKZF1</i>	ankyrin repeat and zinc finger domain containing 1	NM_018089.1	0.036	-0.85	1.81
<i>ARID5A</i>	AT rich interactive domain 5A (MRF1-like)	NM_006673.2	0.042	-0.92	1.89
<i>KLF11</i>	Kruppel-like factor 11	NM_003597.4	0.039	-0.92	1.89
<i>CREB5^b</i>	cAMP responsive element binding protein 5	NM_004904.1	0.010	-1.00	2.00
<i>PNRC1^a</i>	proline-rich nuclear receptor coactivator 1	NM_006813.1	0.008	-1.05	2.07
<i>ATF3</i>	activating transcription factor 3	NM_004024.2	0.049	-1.10	2.14
<i>MAF</i>	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	NM_005360.2	0.044	-1.19	2.29
<i>ZNF385^b</i>	zinc finger protein 385	NM_015481.1	0.011	-1.38	2.61
Cell Adhesion					
<i>HAPLN1^a</i>	hyaluronan and proteoglycan link protein 1	NM_001884.2	0.005	3.00	7.99
<i>CIB1^b</i>	calcium and integrin binding 1 (calmyrin)	NM_006384.2	0.035	0.32	1.25
<i>FLRT2</i>	fibronectin leucine rich transmembrane protein 2	NM_013231.3	0.042	-0.64	1.56
<i>CNTNAP1</i>	contactin associated protein 1	NM_003632.1	0.041	-0.72	1.64
<i>CSNK2A1</i>	casein kinase 2, α 1 polypeptide	NM_177560.2	0.030	-0.73	1.66
<i>LGALS3BP^b</i>	lectin, galactoside-binding, soluble, 3 binding protein	NM_005567.2	0.014	-0.89	1.85
<i>PERP</i>	PERP, TP53 apoptosis effector	NM_022121.2	0.042	-0.92	1.89
<i>ADAM8^b</i>	a disintegrin and metalloproteinase domain 8	NM_001109.1	0.044	-1.06	2.08
<i>CLDN15^b</i>	claudin 15	NM_014343.1	0.022	-1.13	2.19
<i>ADAM33</i>	ADAM metalloproteinase domain 33	NM_153202.1	0.027	-1.22	2.33
<i>PDGFRA</i>	platelet-derived growth factor receptor, α polypeptide	NM_006206.2	0.045	-1.34	2.53

Continued

TABLE 3—Continued

<i>TNXB^b</i>	tenascin XB	NM_032470.2	0.015	−2.32	4.98
<i>DKFZp434B1231^b</i>	eEF1A2 binding protein	NM_178275.3	0.010	−2.66	6.31
Extracellular matrix component, biosynthesis					
<i>HAPLN1^a</i>	hyaluronan and proteoglycan link protein 1	NM_001884.2	0.005	3.00	7.99
<i>TIMP3^a</i>	TIMP metalloproteinase inhibitor 3	BG104808.1	0.006	0.42	1.34
<i>BMP1^b</i>	bone morphogenetic protein 1	NM_006129.2	0.024	−0.62	1.54
<i>B4GALT7^b</i>	xylosylprotein β 1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase I)	NM_007255.1	0.026	−0.66	1.57
<i>MATN2</i>	matrilin 2	NM_030583.1	0.027	−0.96	1.95
<i>ChGn^a</i>	chondroitin beta1,4 N-acetylgalactosaminyltransferase	NM_018371.3	0.015	−1.42	2.68
<i>TNXB^b</i>	tenascin XB	NM_032470.2	0.015	−2.32	4.98
MAPK signaling pathway					
<i>DUSP2^b</i>	dual specificity phosphatase 2	NM_004418.2	0.004	1.17	2.25
<i>STMN1^b</i>	stathmin 1/oncoprotein 18	NM_203401.1	0.006	1.11	2.16
<i>PLA2G12A</i>	phospholipase A2, group XIIA	NM_030821.3	0.044	0.39	1.31
<i>PDE6G^b</i>	phosphodiesterase 6G, cGMP-specific, rod, gamma	NM_002602.1	0.003	−0.53	1.44
<i>TNIP2^b</i>	TNFAIP3 interacting protein 2	NM_024309.2	0.008	−0.62	1.54
<i>UCN</i>	urocortin	NM_003353.2	0.044	−0.65	1.57
<i>CASP7^b</i>	caspase 7, apoptosis-related cysteine peptidase	NM_001227.2	0.023	−0.65	1.57
<i>MAP3K6</i>	mitogen-activated protein kinase kinase kinase 6	NM_004672.3	0.041	−0.67	1.59
<i>BCL3</i>	B-cell CLL/lymphoma 3	NM_005178.2	0.039	−0.79	1.73
<i>MAPK8IP1</i>	mitogen-activated protein kinase 8 interacting protein 1	NM_005456.2	0.037	−0.86	1.81
<i>SPTBN1</i>	spectrin, beta, non-erythrocytic 1 (SPTBN1)	NM_003128.1	0.049	−0.87	1.83
<i>KLF11</i>	Kruppel-like factor 11	NM_003597.4	0.039	−0.92	1.89
<i>PNRC1^a</i>	proline-rich nuclear receptor coactivator 1	NM_006813.1	0.008	−1.05	2.07
<i>PDGFRA</i>	platelet-derived growth factor receptor, α polypeptide	NM_006206.2	0.045	−1.34	2.53
<i>PIK3C2B^a</i>	phosphoinositide-3-kinase, class 2, β polypeptide	NM_002646.2	0.004	−1.54	2.91
<i>RARRES3^b</i>	retinoic acid receptor responder (tazarotene induced) 3	NM_004585.2	0.022	−1.64	3.12

^aGenes within the list of 120 genes identified as differentially expressed in at least 50 percent of the “leave-one-out” datasets.

^bThose within the list of 25 genes identified as differentially expressed in 100 percent of the “leave-one-out” datasets (see Methods for details).

expression profile of periosteal cells from a large group of AS patients (p.Ser252Trp mutation) as compared with normal FGFR2 expressing cells.

Our study represents the largest sample where a dominant gain-of-function mutation of a rare Mendelian disorder is analyzed through microarray technology. Successful detection of a gene expression signature was achieved possibly because all the patients harbor a common mutation in a tyrosine kinase receptor involved in critical developmental signaling cell pathways that leads to a very homogeneous phenotype. On the other hand, our sample size contrasts with the expression profile studies of complex disorders, such as cancer, which are etiologically very heterogeneous and require an even larger number of individuals in

the analysis. It will be important to verify whether other craniosynostotic conditions with either known or unknown causative mutations are also correlated with specific gene expression signatures.

Lemonnier et al. (12) reported that AS p.Ser252Trp mutation induces striking downregulation of FGFR2 protein in osteoblasts, which was attributed to an increased internalization of the receptor. In contrast, our results indicate that AS periosteal cells have a different pattern of FGFR2 expression, as similar FGFR2 transcript and protein levels were observed between control and AS periosteal cells. These discrepancies may be due to differences between the two cell types used in the studies.

Independent confirmation of AS expression changes identified by our mi-

croarray studies came from three strategies. First we used qRT-PCR to confirm the upregulation of six genes (*STMN1*, *SPAG5*, *RRM2*, *HIP2*, *CENPN*, *EEF1B2*) in AS cell lines. As a second approach, we used control periosteal cells treated with high concentrations of exogenous FGF2 (a specific wild type FGFR2c ligand) to mimic the activation status of mutant FGFR2. We observed over-expression in FGF2-treated cells of the six genes previously confirmed to be upregulated in AS cells through qRT-PCR. Curiously, we observed a change in gene expression of the selected genes only after 24 h of FGF2 treatment, suggesting that their expression is a late event of FGFR2c activation. In addition, these results imply that the genes differentially expressed in the AS cells are involved either directly or

Figure 3. Real-time PCR showing expression levels for six genes identified as upregulated in AS cells by microarray analysis: *STMN1*, *SPAG5*, *RRM2*, *HIP2*, *CENPN*, and *EEF1B2*. The expression levels for these genes are enhanced in AS samples when compared with controls.

indirectly with FGFR2c signaling. It is important to point out that this strategy replicates the microarray data in a biologically analogous system. Indeed, Mansukhani et al. (15) showed that gene expression profiles of a murine osteoblast cell line harboring the p.Ser252Trp mutation could be reproduced by exogenous FGF treatment. These findings also suggest that the mutated FGFR2 may over-activate the normal molecular pathways elicited by wild type receptor instead of inducing novel molecular pathways.

As a third approach to validate our microarray results, we looked at the differentially expressed transcripts for genes known to be involved in the canonical FGFR transduction signaling

pathways. As will be discussed later, we observed the presence of *PIK3C2B* gene, which encodes a protein of the phosphoinositide 3-kinase (PI3K) family, and of several members of the MAPK cascades. One of the important signaling pathways leading to activation of the MAP kinases is through FGFR-PI3K hierarchical cascade. We also observed different expression profiles concerning other genes associated with FGFR2 activation, such as *FLRT2*, a positive regulator of FGFR cascade, and *MITF*, a transcription factor acting downstream of FGFR/MEK/ERK signaling. Therefore, our results (including both laboratory and the leave-one-out statistical analysis) support that the expression signature observed in AS cells do represent a

biological state caused by p.Ser252Trp mutation in FGFR2.

Analysis of the genes differentially expressed in AS cells revealed novel expression networks and cellular processes possibly involved in AS pathogenesis. A previous study reported a gene expression profiling experiment using fibroblast periosteal cells obtained from one AS patient (p.Pro253Arg), and two controls (20). We did not observe overlap between the list of genes identified in that study and the set of 263 genes reported here. We think that the very small number of samples analyzed in the previous study (one AS patient and two controls) may have introduced a considerable bias due to individual variation in gene expression, which

Figure 4. Real-time PCR showing expression levels for six genes (*STMN1*, *SPAG5*, *RRM2*, *HIP2*, *CENPN*, and *EEF1B2*) in response to FGF2 treatment. The bars are representing the expression levels of each gene before administration of 0.5 percent FBS and FGF2 (0 h), after 24 h of administration of 0.5 percent FBS (0.5 percent FBS) and after addition of 0.5 percent FBS and FGF2 (0.5 percent FBS + FGF2).

would explain the absence of genes in common in the two studies.

The majority of genes upregulated in AS cells are associated with positive regulation of cell proliferation and nucleotide metabolism, suggesting that the activating FGFR2 mutation induces increased periosteal cell proliferation. Indeed, an excessive proliferation in AS cells in comparison to control cells was clearly evident during tissue culture cell expansion although further experiments are needed to confirm this phenotype.

FGFR-MAPK signaling functions as a trigger of fibroblast cell proliferation. However, we observed downregulation of several members of MAPK signaling

cascades (13 out of 16 genes) as well as *PIK3C2B* in AS cells. It is of note that among the three upregulated genes belonging to the MAPK cascades is *DUSP2*, which is associated with inhibition of MAPK signaling by dephosphorylating activated MAPKs. Interestingly, downregulation of PI3K and MAPK signaling pathways have been associated recently with increased stem cell differentiation (26,27). Therefore, it is possible that the AS mutant FGFR2 expressing cells are more committed toward the osteoblast lineage due to downregulation of transcripts associated with PIK3-MAPK signaling networks. However, further experiments to measure transcript/protein levels of components of

the PIK3-MAPK pathway in mutant FGFR2 expressing cells are warranted to confirm the downregulation and the association of this signaling network with the pathophysiology of AS.

We also found downregulation of most of the genes involved in cell adhesion (11 out of 13 genes) and extracellular matrix composition (five out of seven genes) in AS cells, which contrast one previous study that has shown that periosteal AS cells synthesize a greater amount of extracellular matrix components (including glycosaminoglycans, type I and III collagens, and fibronectin) than normal cells (28). In view of this, it would be interesting to test if the suggested reduction in

cell adhesion and extracellular matrix complexity of our AS periosteal cells are associated with the greater osteogenic capacity of these cells.

As we are aware, the number of significantly altered genes (263 with a $P \leq 0.05$) does not exceed the expected number of genes that would be found by chance in the dataset using the same confidence interval (476 genes out of 9,526 by chance alone at $P \leq 0.05$, and a one-tailed distribution). However, if we took into account the 120 more significant genes (identified as differentially expressed in at least 50 percent of the “leave-one-out” datasets), we did not observe great differences in the distribution of the upregulated and downregulated genes among the functional categories previously discussed for the 263 genes. The exception is the group of genes associated with apoptosis, in which a tendency of enhancement of the number of genes downregulated in AS cells as compared with control cells was observed. These results thus confirm that the most abundant classes of upregulated genes are those related to positive regulation of cell proliferation and nucleotide metabolism, whereas the genes associated with all the other functional categories are mostly downregulated in AS cells.

It was reported that under osteogenic differentiation condition, murine calvarial osteoblast cells heterozygous for p.Ser252Trp mutation in FGFR2 showed increased apoptosis (13,15,16). However, we found an inclination toward downregulation of genes associated with apoptosis between control and AS cells among the 120 genes referred above; in fact, we did not observe significant cell death either in control or in the mutant cell lineages. These discrepancies may be due to differences in the experimental models and cell types used in each study.

Taken together, our findings have important implications in understanding the periosteum function in the postnatal pathogenesis of Apert syndrome patients. The increased proliferative and osteogenic potentials here observed in the AS cells may lead to an expansion of

periosteal cells with the potential to differentiate and contribute to premature suture ossification. It is important to note that a similar mechanism already has been discussed for osteoblast cells upon FGFR over-activation (15). Injuries at the suture site, as those induced by surgical repair, could trigger this abnormal periosteal cell behavior and lead to acceleration of the suture fusion after surgery. Thus, our results suggest for the first time that the mutant periosteal cells in AS patients might play an important role in the pathogenesis of this disease as well as in the recurrent suture closure after surgery.

Recently, identification of mutations in *ephrin-B1* gene associated with craniosynostosis have led to the hypothesis that alteration in the cellular pathways activated by these molecules can lead to an abnormal compartmentalization of the cells during embryogenesis leading to disturbed tissue boundary formation (29). Considering the enhanced osteogenic potential of the AS periosteal cells and also that ephrins recently were shown to activate FGFRs (30), we would like to suggest that a similar mechanism may contribute to the craniosynostosis in FGFR-mutated patients, once the signals that determine separate identities of the cranial suture tissues have been disturbed.

While the full spectrum of molecular factors that modulate these aberrant cellular behaviors remains to be determined, our gene expression profiling study shall contribute to the identification of novel genes with important roles in ossification of cranial sutures or as candidates for syndromic craniosynostosis with still unidentified cause. Of particular note is the recent finding showing that loss of *Dusp6*, a member of the DUSP (dual-specificity phosphatases) family which is activated by FGFRs and inhibits MAP Kinases, cause coronal craniosynostosis in mice (31). Therefore DUSP family members may be considered good candidate genes for FGFR-like craniosynostosis. Interestingly, we found that *DUSP2* is one of the most significant differentially expressed genes in AS periosteal cells. Finally, the understanding of the molecular

pathways involved in the abnormal AS periosteum behavior will certainly have important implications in the prognostic of surgical repair in syndromic craniosynostosis.

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REFERENCES

1. Cohen MM Jr et al. (1992) Birth prevalence study of the Apert syndrome. *Am. J. Med. Genet.* 42: 655–9.
2. Upton J, Zuker RM (eds) (1991) *Clinics in plastic surgery*. Vol 18: Apert syndrome. WB Saunders, Philadelphia.
3. Park WJ et al. (1995) Analysis of phenotypic features and FGFR2 mutations in Apert syndrome. *Am. J. Hum. Genet.* 57:321–8.
4. Slaney SE, et al. (1996) Differential effects of FGFR2 mutations on syndactyly and cleft palate in Apert syndrome. *Am. J. Hum. Genet.* 58:923–32.
5. Cohen MM Jr. (2000) Syndromes with craniosynostosis. In: Cohen Jr MM, MacLean RE. (eds) *Craniosynostosis: Diagnosis, Evaluation, and Management*. New York: Oxford University Press. 309–441.
6. Moloney DM et al. (1996) Exclusive paternal origin of new mutations in Apert syndrome. *Nat. Genet.* 13:48–53.
7. Wilkie AO et al. (1995) Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nat. Genet.* 9:165–72.
8. Anderson J, Burns HD, Enriquez-Harris P, Wilkie AO, Heath JK. (1998) Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. *Hum. Mol. Genet.* 7:1475–83.
9. Yu K, Herr AB, Waksman G, Ornitz DM. (2000) Loss of fibroblast growth factor receptor 2 ligand-

- binding specificity in Apert syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 7:14536–41.
10. Ibrahimi OA, Zhang F, Hrstka SC, Mohammadi M, Linhardt RJ. (2004) Kinetic model for FGF, FGFR, and proteoglycan signal transduction complex assembly. *Biochemistry*. 43:4724–30.
 11. Lomri A, Lemonnier J, Hott M, et al. (1998) Increased calvaria cell differentiation and bone matrix formation induced by fibroblast growth factor receptor 2 mutations in Apert syndrome. *J. Clin. Invest.* 101:1310–7.
 12. Lemonnier J, Delannoy Ph, Hott M, Lomri A, Modrowski D, Marie PJ. (2000) The Ser252Trp fibroblast growth factor receptor-2 (FGFR-2) mutation induces PKC-independent downregulation of FGFR-2 associated with premature calvaria osteoblast differentiation. *Exp. Cell Res.* 256:158–67.
 13. Mansukhani A, Bellosta P, Sahni M, Basilico C. (2000) Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. *J. Cell Biol.* 149:1297–1308.
 14. Lomri A, Lemonnier J, Delannoy P, Marie PJ. (2001) Increased expression of protein kinase Calpha, interleukin-1alpha, and RhoA guanosine 5'-triphosphatase in osteoblasts expressing the Ser252Trp fibroblast growth factor 2 receptor Apert mutation: identification by analysis of complementary DNA microarray. *J. Bone Miner. Res.* 16:705–12.
 15. Mansukhani A, Ambrosetti D, Holmes G, Cornivelli L, Basilico C. (2005) Sox2 induction by FGF and FGFR2 activating mutations inhibits Wnt signaling and osteoblast differentiation. *J. Cell Biol.* 168:1065–76.
 16. Chen L, Li D, Li C, Engel A, Deng CX. (2005) A Ser252Trp [corrected] substitution in mouse fibroblast growth factor receptor 2 (Fgfr2) results in craniosynostosis. *Bone*. 33:169–78. Erratum in: *Bone*. (2005) 37:876.
 17. Wang Y et al. (2005) Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/-S252W) mouse. *Development*. 132:3537–48.
 18. Hopper RA et al. (2001) Effect of isolation of periosteum and dura on the healing of rabbit calvarial inlay bone grafts. *Plast. Reconstr. Surg.* 107:454–62.
 19. Ozerdem OR et al. (2003) Roles of periosteum, dura, and adjacent bone on healing of cranial osteonecrosis. *J. Craniofac. Surg.* 14:371–9.
 20. Carinci F et al. (2002) Expression profiles of craniosynostosis-derived fibroblasts. *Mol. Med.* 8:638–44.
 21. Zuk PA et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* 7:211–28.
 22. Quackenbush J. (2002) Microarray data normalization and transformation. *Nat Genet.* 32(Suppl.):496–501.
 23. Golub TR et al. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*. 286:531–7.
 24. Reis EM et al. (2004) Antisense intronic non-coding RNA levels correlate to the degree of tumor differentiation in prostate cancer. *Oncogene*. 23:6684–92.
 25. van't Veer LJ et al. (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 415:530–6.
 26. Takahashi K, Murakami M, Yamanaka S. (2005) Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells. *Biochem. Soc. Trans.* 33:522–5.
 27. Armstrong L et al. (2006) The role of PI3K/AKT, MAPK/ERK and NFkappabeta signaling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. *Hum. Mol. Genet.* 15:1894–1913.
 28. Bodo M et al. (1997) Glycosaminoglycan metabolism and cytokine release in normal and otosclerotic human bone cells interleukin-1 treated. *Eur. J. Clin. Invest.* 27:36–42.
 29. Twigg SR et al. (2004) Mutations of ephrin-B1 (EFNB1), a marker of tissue boundary formation, cause craniofrontonasal syndrome. *Proc. Natl. Acad. Sci. U S A.* 101:8652–7.
 30. Yokote H et al. (2005) Trans-activation of EphA4 and FGF receptors mediated by direct interactions between their cytoplasmic domains. *Proc. Natl. Acad. Sci. U. S. A.* 102:8866–71.
 31. Li C, Scott DA, Hatch E, Tian X, Mansour SL. (2007) Dusp6 (Mkp3) is a negative feedback regulator of FGF-stimulated ERK signaling during mouse development. *Development*. 134:167–76.

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Genetics of Craniosynostosis: Genes, Syndromes, Mutations and Genotype-Phenotype Correlations

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Abstract

Craniosynostosis is a very heterogeneous group of disorders, in the etiology of which genetics play an important role. Chromosomal alterations are important causative mechanisms of the syndromic forms of craniosynostosis accounting for at least 10% of the cases. Mutations in 7 genes are unequivocally associated with mendelian forms of syndromic craniosynostosis: *FGFR1*, *FGFR2*, *FGFR3*, *TWIST1*, *EFNB1*, *MSX2* and *RAB23*. Mutations in 4 other genes, *FBNI*, *POR*, *TGFBR1* and *TGFBR2*, are also associated with craniosynostosis, but not as the major clinical feature of the phenotype or with an apparently low penetrance. The identification of these genes represented a great advance in the dissection of the genetics of craniosynostosis in the last 15 years, and today they explain the etiology of about 50% of the syndromic cases. The paucity in the identification of genes associated with this defect has partly been due to the rarity of familial cases. In contrast, very little is known about the molecular and cellular factors leading to nonsyndromic forms of craniosynostosis. Revealing the molecular pathology of craniosynostosis is also of great value for diagnosis, prognosis and genetic counseling. This chapter will review (1) the chromosomal regions associated with syndromic forms of the malformation, (2) the genes in which a large number of mutations have been reported by independent studies (*FGFR1*, *FGFR2*, *FGFR3*, *TWIST1* and *EFNB1*) and (3) the molecular mechanisms and genotype-phenotype correlations of such mutations.

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Introduction

Cranial suture fusion occurs at specific periods during the lifetime of an individual, and therefore, abnormal activation of cell signaling triggered by genetic and/or environmental factors during embryogenesis or early childhood

can alter the patency of the sutures. The two most common processes associated with cranial suture developmental defects are craniosynostosis, premature fusion of cranial sutures, and parietal foramina, delayed differentiation of the bones of the skull. Although common genes seem to be involved with these two cranial suture abnormalities, this chapter will focus on the genetics of craniosynostosis.

The prevalence of craniosynostosis is estimated to be 1 to 2,000–3,000 births [1]. The frequencies of different types of craniosynostosis vary according to ascertainment centers, but on average, of all craniosynostosis, sagittal synostosis is the most common (40–55%), followed by coronal (20–25%), metopic (5–15%), multiple suture synostosis (5–15%) and lambdoid (0–5%) [1].

Craniosynostosis has usually been classified as nonsyndromic (or isolated) and syndromic forms for genetic studies. Nonsyndromic craniosynostosis accounts for 70% of the cases [2–4], and occurs when cranial suture fusion is the only primary defect in the individual. Secondary symptoms, such as neurologic or ophthalmologic manifestations, can be present as a consequence of early sutural obliteration [1]. On the other hand, syndromic craniosynostosis occurs associated with other primary defects of morphogenesis. In practice, it is very hard to make this distinction and it is possible that some nonsyndromic forms actually represent the end of the spectrum of the clinical variability of syndromic forms.

Familial recurrence is reported for 14% in nonsyndromic coronal synostosis, 6% in sagittal synostosis, 3–9% in metopic synostosis and 22% in the syndromic ones [2–4, Jehee, unpubl. data]. Pedigrees from familial cases are compatible with autosomal dominant, autosomal recessive or X-linked inheritance [1–5]. Multifactorial inheritance also seems to play a role in the etiology of the nonsyndromic forms of craniosynostosis, although additional epidemiological studies should be performed.

The genetic etiology of the nonsyndromic craniosynostosis is still very poorly understood: to date, *EFNA4* is the only gene that when mutated causes only nonsyndromic craniosynostosis [6]. However, the effect of mutations in this gene on the human phenotype still depends on the identification of a larger number of patients with *EFNA4* pathogenic changes. Conventional karyotype analysis is not recommended for nonsyndromic craniosynostosis.

Despite the lower proportion of syndromic forms, their genetic analysis has largely contributed to the elucidation of some important pathways for suture development and closure. There are at least 150 syndromes associated with craniosynostosis as a major clinical feature. Mendelian and chromosomal alterations are important causative mechanisms of this group of craniosynostosis. Linkage analysis in familial cases and molecular analysis of chromosomal alterations have led to the identification of six genes that when mutated are unequivocally associated with syndromic craniosynostosis: *FGFR1*, *FGFR2*, *FGFR3*, *TWIST1*, *EFNB1*, *MSX2* (table 1). More recently *RAB23* has been

Table 1. Genes and phenotypes associated with craniosynostosis

Gene	Gene symbol	Chromosome	Phenotypes	MIM	Penetrance craniosynostosis
Fibroblast growth factor receptor 1	<i>FGFR1</i>	8p11.2-p11.1	Pfeiffer osteoglyphonic dysplasia	101600	high ^a
Fibroblast growth factor receptor 2	<i>FGFR2</i>	10q26	Crouzon Crouzon with scaphocephaly Jackson-Weiss Pfeiffer Apert SCS like cutis gyrata syndrome of Beare-Stevenson	166250 123500 123150 101600 101200 101400 123790	apparently high high ^b high high high high high
			Antley-Bixley ^c nonclassifiable syndromes with craniosynostosis nonsyndromic coronal synostosis	207410	apparently high ^b
Fibroblast growth factor receptor 3	<i>FGFR3</i>	4p16.3	Muenke syndrome Crouzon with acanthosis nigricans SCS like thanatophoric dysplasia type I thanatophoric dysplasia type II SCS nonsyndromic	602849 123500 101400 187600 187601 101400	high high high low (<30%) high high
Twist homolog Drosophila 1	<i> Twist1</i>	7p21	nonsyndromic craniosynostosis	very few cases	reported
Ephrin-B1	<i>EFNB1</i>	Xq12	craniofrontonasal syndrome	304110	high
Ras-associated protein RAB23	<i>RAB23</i>	6p11	Carpenter syndrome	201000	high
Muscle segment homeobox homolog Drosophila 2	<i>MSX2</i>	5q34-35	craniosynostosis Boston type	604757	high

Table 1. (continued)

Gene	Gene symbol	Chromosome	Phenotypes	MIM	Penetrance craniosynostosis
Transforming growth factor- β receptor type I	<i>TGFBRI</i>	9q33-q34	Loeys-Dietz syndrome	609192	low (<30%)
Transforming growth factor- β receptor type II	<i>TGFBRII</i>	3p22	Loeys-Dietz syndrome		low (<30%)
Cytochrome P450 reductase gene	<i>POR</i>	7q11.2	Antley-Bixley	207410	apparently high
Fibrillin	<i>FBN1</i>	15q21	Shprintzen-Goldberg craniosynostosis syndrome	182212	very few cases reported

^aHigh = Penetrance higher than 70%.

^bVariability of the phenotype possibly related to the type of mutation.

^cUnder discussion.

included in this list [7]. Mutations in 4 other genes, *FBNI*, *POR*, *TGFBR1* and *TGFBR2* (table 1), are also associated with craniosynostosis, but not as the major clinical feature of the phenotype and/or with an apparently low penetrance [8–10]. The identification of these genes represented a great advance in the dissection of the genetics of craniosynostosis in the last 15 years, even though they explain the etiology of about 50% of the syndromic cases. The paucity in the identification of genes associated with this defect has partly been due to the rarity of familial cases.

Revealing the molecular pathology of craniosynostosis has also been of great value for diagnosis, prognosis and genetic counseling. In this chapter we will deal with the genetics of the syndromic forms of craniosynostosis.

Chromosome Alterations in the Etiology of Craniosynostosis

All types of chromosomal abnormalities have already been described in patients with craniosynostosis, including deletions and duplications in almost all human chromosomes (fig. 1). The large number of chromosomal alterations and their ubiquitous location in the human genome indicate the vast genetic heterogeneity of this condition and reinforce the importance of performing karyotype analysis in patients with syndromic craniosynostosis that have been excluded for any of the known monogenic forms. It was estimated that approximately 16% of all cases of syndromic craniosynostosis are related to chromosomal abnormalities [11]. There is a high association of craniosynostosis with duplication 13q21-q34 and deletion 7p15-p21, 9p21-p24 and 11q23-q25 [50, 51]. Furthermore, an increasing number of craniosynostotic patients with deletion 22q11 and deletion and or duplication 1p36 have been reported [12, 33].

The mapping of important genes involved in suture closure based on chromosomal abnormalities has been limited. With the exception of 7p21, 9p21 and 11q23 deletions, the number of cases carrying each chromosomal alteration is not large enough to enable phenotype/genotype correlations. Furthermore, the presence of craniosynostosis is not the same in all patients bearing the same chromosomal rearrangement; for instance, around 65–100% of patients with deletions 9p21 and only 50–70% of patients with deletions 11q23 present metopic synostosis [52–55]. This suggests that other factors such as the environmental and genetic background of the individual could be important for the penetrance of craniosynostosis.

Fig. 1. Human chromosomal map depicting duplications and deletions associated with craniosynostosis [data based on 1, 12–49].

Mendelian Causes of Craniosynostosis

Herein we include a detailed description of the genes *FGFR1*, *FGFR2*, *FGFR3*, *TWIST1* and *EFNB1*, as several mutations in each of these genes have been associated with autosomal dominant forms of craniosynostosis, mostly syndromic forms as illustrated in figure 2.

Fibroblast Growth Factor Receptors (FGFRs)

FGFR Structure and Functions

The FGFR family consists of four closely related members of signal-transduction receptor tyrosine kinases (FGFR1–4). Each receptor is composed of three extracellular immunoglobulin-like domains (IgI, IgII and IgIII), a single-pass

Fig. 2. Facial and limb appearance of patients with craniosynostosis. **a** Pfeiffer syndrome, FGFR1 p.Pro252Arg (courtesy of Dr. N. Alonso). **b–d** Pfeiffer syndrome, FGFR2 c.940-1G>C. **e** Pfeiffer syndrome, FGFR2 p.Trp290Cys. **f, g** Apert syndrome, FGFR2 p.Pro253Arg. **h–j** Apert syndrome, FGFR2 p.Ser252Trp. **k** Crouzon syndrome, FGFR2 p.Cys278Phe. **l–n** Three affected members of the same family, Muenke syndrome, FGFR3 p.Pro205Arg. **o–q** SCS, 46,XX, ins(7;9)(p21.2;p21.2p24.2). **r** SCS, TWIST1 c.385_405Ile135insAALRKII.

transmembrane domain (TM), and a split intracellular tyrosine kinase domain (TK1/TK2). The IgII and IgIII loops are critical for fibroblast growth factors (FGF) binding. The formation of a complex involving two FGFs, a FGFR dimer and cell surface heparan sulfate proteoglycans is required for autophosphorylation of various tyrosine residues in the TK domain [56]. Once phosphorylated, these tyrosines initiate signal transduction through a diverse array of signaling pathways, which may control phenomena such as cell proliferation, differentiation, migration and apoptosis according to the context [57, 58].

Complexity in FGFR signaling is enhanced by a wide array of alternative splicings. Most important is the alternative splicing of the exons encoding the C-terminal half of the third Ig loop of FGFR1–3. For these three receptors, the IgIII loop is encoded by two exons, an invariant exon termed IIIa and one of two exons, termed IIIb and IIIc, respectively, to which the IIIa exon is spliced. This generates two receptor isoforms with different ligand-binding specificities [59]. Best known are the variants of FGFR2: FGFR2b (encoded by the IIIb exon) is expressed mainly on the epithelia and is activated by ligands synthesized predominantly in the tissue mesenchyme; on the other hand, FGFR2c (encoded by the IIIc exon), the most abundant one, is located primarily in the mesenchyme and preferentially recognizes epithelial FGFs.

FGF-FGFR signaling plays a critical role in early embryonic development and in organogenesis and heterozygous mutations in the FGFR1–3 have been associated with a number of different dominant disorders, including craniosynostosis (gain-of-function mutations in FGFR1–3) [60–81], short-limbed bone dysplasias

(gain-of-function mutations in *FGFR3*) [60, 82–85], Kallmann syndrome (haploinsufficiency for *FGFR1*) [86], lacrimo-auriculo-dento-digital (LADD) syndrome (possibly due to haploinsufficiency for *FGFR2* and *FGFR3* and dominant-negative *FGF10* mutation) [87] and camptodactyly, tall stature, scoliosis, and hearing loss (CATSHL) syndrome (dominant-negative *FGFR3* mutation) [88]. In this section *FGFR1–3* mutations associated with craniosynostosis are discussed.

Gain-of-Function *FGFR* Mutations Associated with Craniosynostosis: Overview

Heterozygous mutations in *FGFR1*, *FGFR2* and *FGFR3* genes account for the majority of cases of syndromic craniosynostosis. The *FGFRs* craniosynostotic syndromes share several craniofacial features including premature closure of coronal or other cranial sutures, but have distinct limb and dermatological features. In addition, some patients with nonclassifiable craniosynostotic syndromes or with nonsyndromic craniosynostosis have been shown to bear mutations in *FGFR2* and *FGFR3* genes (tables 1, 2).

The *FGFR1–3* mutations associated with craniosynostosis identified so far are summarized in table 2 and illustrated in figure 3 [60–81]. All pathogenic *FGFR1–3* mutations associated with craniosynostosis act dominantly and confer gain-of-function to the mutated receptor through different mechanisms, including (1) FGF-independent receptor dimerization and activation, (2) enhanced FGF-binding affinity, (3) loss of FGF-binding specificity and (4) ectopic splice form expression.

FGFR-related craniosynostoses are found both in familial and sporadic cases, and some mutations are highly recurrent. An exclusive paternal origin of de novo mutations has been described for several *FGFRs* mutations [89, 90].

Genotype-Phenotype Correlations

Analysis of *FGFR1–3* mutations reveals a complex pattern of phenotype-genotype correlations. This is further complicated by the fact that the distinction between the craniosynostosis syndromes is not absolute and clinical overlapping is a common feature. There are several examples in which clinically different phenotypes can be caused by either the same mutation or by equivalent mutations on each of the *FGFR1–3*, while a given phenotype can be associated with many different mutations in the same or different *FGFRs*. In addition, low penetrance mutations have also been observed (table 2).

Table 2. *FGFR1–3* mutations associated with craniosynostosis

Gene	Nucleotide change ^a	Effects on protein or RNA ^a	Gene or protein region ^b	Phenotype	Reference No. ^c
<i>FGFR 1</i>	c.755C>G	p.Pro252Arg	IgII-IgIII linker	Pfeiffer syndrome; Jackson-Weiss syndrome	61
	c.929T>A	p.Asn330Ile	IgIIIc	osteoglophonic syndrome	62
	c.1115G>A	p.Tyr372Cys	IgIII-TM	osteoglophonic syndrome	62
	c.1135T>C	p.Cys379Arg	TM	osteoglophonic syndrome	62
<i>FGFR 2</i>	c.314A>G	p.Tyr105Cys	IgI	Crouzon syndrome	
	c.514-515GC>TT	p.Ala172Phe	IgII	Pfeiffer syndrome	
	c.755C>G	p.Ser252Trp	IgII-IgIII linker	Apert syndrome, Pfeiffer syndrome	
	c.755C>T	p.Ser252Leu ^d	IgII-IgIII linker	Crouzon syndrome	
	c.755 - 756 CG>TC	p.Ser252Phe	IgII-IgIII linker	Apert syndrome	
	c.755 - 757 CGC>TCT	p.Ser252Phe/Pro253Ser	IgII-IgIII linker	Pfeiffer syndrome	
	c.758C>G	p.Pro253Arg	IgII-IgIII linker	Apert syndrome	
	c.758C>T	p.Pro253Leu	IgII-IgIII linker	Crouzon syndrome	63
	c.760C>T	p.His254Tyr	IgII-IgIII linker	Crouzon syndrome	
	c.788C>T	p.Pro263Leu	IgII-IgIII linker	Crouzon syndrome	
	c.799T>C	p.Ser267Pro	IgII-IgIII linker	Crouzon syndrome, Pfeiffer syndrome	
	c.803insTGG	p.Thr268ThrGly	IgIIIa	Crouzon syndrome	
	c.804_809delGTGGTC	p.Val269_Val270del	IgIIIa	nonclassifiable disorder with craniosynostosis	
	c.818_820del	p.Asp273del	IgIIIa	Pfeiffer syndrome	64
	c.823_824ins12	p.Val274_Glu275ins4	IgIIIa	Crouzon syndrome	65
	c.826T>G	p.Phe276Val	IgIIIa	Crouzon syndrome, Pfeiffer syndrome	

Table 2. (continued)

Gene	Nucleotide change ^a	Effects on protein or RNA ^a	Gene or protein region ^b	Phenotype	Reference No. ^c
	c.833G>A	p.Cys278Tyr ^d	IgIIIa	Crouzon syndrome; nonsyndromic sagittal/unilambdoid synostosis	66
	c.833G>T	p.Cys278Phe	IgIIIa	Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome	67
	c.842A>G	p.Tyr281Cys	IgIIIa	Crouzon syndrome	
	c.858_866delCCACATCCA	p.His287_Gln ^d del	IgIIIa	Crouzon syndrome	68
	c.863T>A	p.Ile288Asn	IgIIIa	Crouzon syndrome	
	c.863T>G	p.Ile288Ser	IgIIIa	Crouzon syndrome	
	c.864_881del	p.Ile288Met; Gln289_Val294del	IgIIIa	Pfeiffer syndrome	
	c.866A>C	p.Gln289Pro	IgIIIa	Crouzon syndrome, Jackson-Weiss syndrome, SCS	69, 70
	c.868T>C	p.Trp290Arg	IgIIIa	Crouzon syndrome	
	c.868T>G	p.Trp290Gly	IgIIIa	Crouzon syndrome	
	c.870G>C	p.Trp290Cys	IgIIIa	Pfeiffer syndrome	
	c.870G>T	p.Trp290Cys	IgIIIa	Pfeiffer syndrome, nonclassifiable disorders with craniosynostosis	
	c.874A>G	p.Lys292Glu	IgIIIa	Crouzon syndrome	
	c.902A>G	p.Tyr301Cys	IgIIIa	Crouzon syndrome	68
	c.923A>G	p.Tyr308Cys	IgIIIa	Crouzon syndrome	
	c.940G>T	p.Ala314Ser	IgIIIa	Pfeiffer syndrome	
	c.940-1G>A	splicing	intron 9 – splice acceptor	Pfeiffer syndrome	
	c.940-1G>C	splicing	intron 9 – splice acceptor	Pfeiffer syndrome	
	c.940-2A>G ^e	splicing	intron 9 – splice acceptor	Pfeiffer syndrome; Apert syndrome	
	c.940-2A>T ^e	splicing	intron 9 – splice acceptor	Pfeiffer syndrome	

c.940-3T>G	splicing	intron 9 – splice acceptor	Pfeiffer syndrome	
c.940-3_-4insAlu ^e	splicing	intron 9	Apert syndrome	
c.940-3_946del10insACC	splicing	intron 9	Pfeiffer syndrome	
c.943G>T	p.Ala315Ser ^d	IgIIIc	nonsyndromic unicoronal synostosis	68
c.958_959delAC ^e	p.Thr320GlyfsX5	IgIIIc	Jackson-Weiss syndrome	
c.966A>C	p.Asp321Ala	IgIIIc	Pfeiffer syndrome	
c.983A>G	p.Tyr328Cys	IgIIIc	Crouzon syndrome	
c.992A>T	p.Asn331Ile	IgIIIc	Crouzon syndrome	
c.1009G>A	p.Ala337Thr ^d	IgIIIc	nonsyndromic unicoronal synostosis	71
c.1009G>C	p.Ala337Pro	IgIIIc	Crouzon syndrome	
c.1011-1012insGACGCT	p.AspAla377_378ins	IgIIIc	Crouzon syndrome	
c.1012G>C	p.Gly338Arg	IgIIIc	Crouzon syndrome	
c.1013G>A	p.Gly338Glu	IgIIIc	Crouzon syndrome	
c.1018T>C	p.Tyr340His	IgIIIc	Crouzon syndrome	
c.1019A>C	p.Tyr340Ser	IgIIIc	Crouzon syndrome	68
c.1019A>G	p.Tyr340Cys	IgIIIc	Pfeiffer syndrome	
c.1021A>C	p.Thr341Pro	IgIIIc	Pfeiffer syndrome	
c.1024T>A	p.Cys342Ser	IgIIIc	Crouzon syndrome, Jackson-Weiss syndrome, Pfeiffer syndrome	72
c.1024T>C	p.Cys342Arg	IgIIIc	Crouzon syndrome, Pfeiffer syndrome	
c.1024T>G	p.Cys342Gly	IgIIIc	Crouzon syndrome, Jackson-Weiss syndrome, Pfeiffer syndrome	73
c.1025G>A	p.Cys342Tyr	IgIIIc	Pfeiffer syndrome	
c.1025G>C	p.Cys342Ser	IgIIIc	Crouzon syndrome, Pfeiffer syndrome	
c.1025G>T	p.Cys342Phe	IgIIIc	Crouzon syndrome, Jackson-Weiss syndrome, Pfeiffer syndrome	74
c.1025_1026GC>CT	p.Cys342Ser	IgIIIc	Crouzon syndrome, Pfeiffer syndrome	
c.1026C>G	p.Cys342Trp	IgIIIc	Crouzon syndrome, Pfeiffer syndrome	74
c.1030G>C	p.Ala344Pro	IgIIIc	Pfeiffer syndrome	
c.1031C>G	p.Ala344Gly	IgIIIc	Jackson-Weiss syndrome, Crouzon syndrome	

Table 2. (continued)

Gene	Nucleotide change ^a	Effects on protein or RNA ^a	Gene or protein region ^b	Phenotype	Reference No. ^c
	c.1032G>A ^f	p.Ala344Ala (Splicing) ^d	IgIIIc	Crouzon syndrome; nonclassifiable disorders with craniosynostosis	
	c.1040C>G	p.Ser347Cys splicing	IgIIIc	Crouzon syndrome	
	c.1041_1042insAlu ^e		IgIIIc	Apert syndrome	
	c.1052C>G	p.Ser351Cys	IgIIIc	Crouzon syndrome, Pfeiffer syndrome, unclassified	75, 76
				craniosynostosis	
	c.1059C>A	p.Ser354Tyr	IgIIIc	Crouzon syndrome	66
	c.1061C>A	p.Ser354Tyr	IgIIIc	Crouzon syndrome	
	c.1061C>G	p.Ser354Cys	IgIIIc	Crouzon syndrome	
	c.1061C>T	p.Ser354Phe	IgIIIc	Crouzon syndrome	
	c.1066-1074delITGGTTTGACA	p.Trp356_Thr358del	IgIIIc	Crouzon syndrome	
	c.1075G>T	p.Val359Phe	IgIIIc	Pfeiffer syndrome; Jackson-Weiss syndrome	74
	c.1084G>T	p.Ala362Ser ^d	IgIIIc	Crouzon syndrome	77
	c.1084+1G>T	splicing	intron 10 – splice donor	Pfeiffer syndrome	
	c.1084+3A>C	splicing	intron 10 – splice donor	Crouzon syndrome	66
	c.1084+3A>G	splicing	intron 10 – splice donor	Pfeiffer syndrome	
	c.1084_1085ins_TCAACA	p.Gly345_Pro361del	IgIIIc	Pfeiffer syndrome	
	c.1115C>G	p.Ser372Cys	IgIIIc-TM	Beare-Stevenson cutis gyrata syndrome	
	c.1124A>G	p.Tyr375Cys	IgIIIc-TM	Beare-Stevenson cutis gyrata syndrome; Pfeiffer syndrome	
	c.1150G>A	p.Gly384Arg	TM	unclassified craniosynostosis	78, 79
	c.1576A>G	p.Lys526Glu ^d	TK1	mild Crouzon syndrome, scaphocephaly	
	c.1645A>C	p.Asn549His	TK1	Crouzon syndrome	
	c.1646A>C	p.Asn549Thr	TK1	Pfeiffer syndrome	63

3	<i>FGFR</i>	c.1694A>C	p.Glu565Ala	TK1	Pfeiffer syndrome	80
		c.1694A>G	p.Glu565Gly	TK1	Pfeiffer syndrome	
		c.1922A>G	p.Lys641Arg	TK2	Pfeiffer syndrome	
		c.1977G>T	p.Lys659Asn	TK2	nonclassifiable syndrome with craniosynostosis	
		c.1988G>A	p.Gly663Glu	TK2	Pfeiffer syndrome	
		c.2032A>G	p.Arg678Gly	TK2	Crouzon syndrome	
		c.742C>T	p.Arg248Cys ^g	IgII-IgIII linker	thanatophoric dysplasia type I	
		c.749C>G	p.Pro250Arg ^d	IgII-IgIII linker	Muenke syndrome; Beare-Stevenson cutis gyrata syndrome	
		c.749C>T	p.Pro250Leu ^d	IgII-IgIII linker	nonsyndromic unicoronal craniosynostosis in child, macrocephaly in mother	
		c.1118A>G	p.Tyr373Cys ^g	IgIII-TM linker	thanatophoric dysplasia type I	81
		c.1172C>A	p.Ala391Glu	TM	Crouzonodermoskeletal syndrome ^h	
		c.1948A>G	p.Lys650Glu ⁱ	TK2	thanatophoric dysplasia type II	

^aNucleotide and amino acid residue numberings are in accordance with Cohen [1, 60] or the original reports. Mutation nomenclature is in accordance with the recommendations of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>).

^bGene region or protein domain affected by the mutation.

^cAll the original reports of the mutations are cited here except for the ones included in Cohen [1, 60].

^dMutations either associated with reduced penetrance or with uncertain pathogenicity

^eMutations that lead to ectopic expression of the alternative FGFR2b splice form.

^fMutation that produces a cryptic donor splice site that causes a 17-amino acid deletion.

^gThanatophoric dysplasia type I mutations more frequently associated with craniosynostosis.

^hAlso referred to as Crouzon syndrome with acanthosis nigricans.

ⁱCraniosynostosis is present in ~93% of thanatophoric dysplasia type II subjects with this mutation.

Fig. 3. Structure of FGFR proteins (types 1, 2 and 3) showing the approximate location of the mutations causing craniosynostosis. AB = Acid box; IgI, IgII, IgIII = immunoglobulin-like domains; IgIIIa = N-terminal portion of the IgIII loop (common to both FGFR1-3b and FGFR1-3c splice forms); IgIIIc = alternatively spliced form of the C-terminal portion of IgIII loop; TM = transmembrane domain; TK1 = first kinase domain; TK2 = second kinase domain.

FGFR1 Mutations

The most common FGFR1-activating mutation is the amino acid substitution p.Pro252Arg in the IgII-IgIII linker region. This heterozygous mutation has been found in a few cases of Pfeiffer syndrome (with a benign course) [60] and in a subject with Jackson-Weiss syndrome [61].

FGFR1 mutations were also found in patients with osteoglophonic dysplasia, a condition characterized by craniosynostosis, a prominent supraorbital ridge, depressed nasal bridge, rhizomelic dwarfism and nonossifying bone lesions [62]. This association reveals the critical role of FGFR1 in the modulation of bone elongation in humans.

Analogous mutations in FGFR2 or FGFR3 have been found for the four gain-of-function mutations in FGFR1 so far identified. Although there is phenotypic overlap among patients, these analogous mutations in different receptors are associated with different clinical entities. For example, FGFR1 p.Pro252Arg substitution is analogous to FGFR2 p.Pro253Arg and FGFR3 p.Pro250Arg that cause Apert and Muenke syndromes, respectively [60]. Another example is FGFR1 p.Asn330Ile mutation in the IgIIIc loop associated with osteoglophonic dysplasia [62], which is equivalent to FGFR2 p.Asn331Ile and FGFR3 p.Asn328Ile that cause Crouzon and hypochondroplasia syndromes, respectively [60].

FGFR2 Mutations

Mutations in FGFR2 account for approximately 90% of the syndromic craniosynostosis Apert, Crouzon, Pfeiffer, and Jackson-Weiss [60, 67, 68, 91] and for 9% of all craniosynostosis [67, 71, 92]. No FGFR2 mutations have been found in any case of nonsyndromic sagittal or metopic synostosis [71].

In contrast to FGFR1 mutations, a broader spectrum of FGFR2 mutations has been associated with craniosynostosis. The majority of FGFR2 mutations are missense (to date about 65 amino acid substitutions reported) or splice-site type (about a dozen mutations reported) but small in-frame deletions and insertions have been also described. Several of the missense mutations (~21%) either create or destroy cysteine residues, resulting in unpaired cysteines that can produce intermolecular disulfide binding and ligand-independent constitutive receptor activation (table 2, fig. 3).

Mutations in the IgI and IgII Loops of FGFR2

Mutations within IgI and IgII domains have only been found in FGFR2 (table 2, fig. 3). The associated phenotypes resemble those with mutations in the hot spots of the *FGFR2* (exons IIIa and IIIc, or exons 8 and 10, respectively, encoding the IgIII domain) but with some unusual clinical features, such as Crouzon syndrome with scaphocephaly [60, 75] in one case and a Pfeiffer phenotype with severe limb abnormalities, including symphalangism [60, 93].

Mutations in the IgII-IgIII Linker Region of FGFR2

The two most frequent mutations in the linker region between IgII and IgIII of FGFR2 are p.Ser252Trp and p.Pro253Arg that cause 66 and 32.2% of

Apert syndrome cases, respectively. The p.Ser252Trp mutation is associated with a more severe craniofacial phenotype with cleft palate in some patients, while p.Pro253Arg mutation is associated with more severe syndactyly [60, 94]. Interestingly, one patient bearing the p.Ser252Trp mutation had such a mild syndactyly that the phenotype suggested Pfeiffer rather than Apert syndrome [60].

Few rare additional mutations in the IgII-IgIII linker region are also associated with Apert and Pfeiffer syndrome. The type of amino acid residue substitution has a specific clinical consequence, as illustrated by the observation of Crouzon-like phenotypes and nonpenetrance of the disease in cases with p.Ser252Leu or p.Pro253Leu substitutions.

Unlike mutations within other regions of FGFRs, the Apert syndrome p.Ser252Trp and p.Pro253Arg mutations are substitutions to bulky side-chain amino acids and are ligand dependent. It has been demonstrated that these substitutions define two gain-of-function mechanisms of FGFR2 mutations: enhanced FGF-binding affinity and loss of FGF-binding specificity [95–97]. Analogous mutations on FGFR1 and FGFR3 seem to behave similarly to the Apert FGFR2 mutations [98].

Mutations in the IgIII loop of FGFR2

The majority of FGFR2 mutations (~77%) are clustered in the IgIII loop of the receptor (table 2, fig. 3). Although several mutations have been associated with more than one syndrome, a genotype-phenotype correlation is observed in many cases. For example, Crouzon syndrome appears to be preferentially accounted for in the case of substitutions at residues p.Phe267, p.Cys278, p.Gln289, p.Gly338, p.Cys342, p.Ala344, p.Ser347 and p.Ser354; severe Pfeiffer syndrome cases, on the other hand, are more frequently associated with mutations at residues p.Trp290, p.Tyr340, p.Cys342 and p.Ser351 when they are substituted by a cysteine or if a cysteine residue is abolished. The residue that is substituted is also important for the phenotype, as exemplified by the conversion of the p.Trp290 and p.Tyr340 into other amino acid residues rather than cysteines (p.Trp290Arg/Gly, p.Tyr340His/Ser) that result in Crouzon syndrome [60, 68, 93].

One interesting category of FGFR2 mutations is the splicing mutations that are mostly associated with Pfeiffer syndrome (accounts for ~10% of mutations associated with this syndrome), and usually with a more severe limb phenotype [60, 93].

Another interesting class of mutations constitutes two *de novo* *Alu*-element insertions upstream or within exon IIIc (or exon 10) in 2 Apert syndrome patients. This type of mutation and a few other pathogenic changes affecting this domain lead to ectopic expression of the FGFR2b splice form in cells of

mesenchyme origin [68, 99]. Therefore, these mutations define a new pathological class of FGFR2 mutations: ectopic splice form expression.

Mutations Either Near or within the Transmembrane Region of FGFR2

Two closely spaced mutations p.Ser372Cys and p.Tyr375Cys, in the linker region between IgIII and the transmembrane domain of FGFR2, have been associated with Beare-Stevenson cutis gyrata syndrome [60]. The latter was also recently found in a severe Pfeiffer phenotype, but in which cutis gyrata and acanthosis nigricans, clinical findings associated with Beare-Stevenson syndrome, were not observed [60, 91].

The only mutation within the transmembrane domain of FGFR2, p.Gly384Arg, has been associated with a nonclassifiable disorder with craniosynostosis [60].

Mutations in the Tyrosine Kinase Domain of FGFR2

A total of nine distinct mutations associated with syndromic craniosynostosis have been identified so far in the FGFR2 tyrosine kinase domain (table 2, fig. 3). There does not appear to be a set of clinical features that uniformly distinguishes patients with FGFR2 intracellular tyrosine kinase mutations from those with mutations in the extracellular region, except that patients tend to exhibit mild broadening of the thumbs and great toes [91, 93].

Some of these mutations occur at equivalent positions of FGFR3, but the associated phenotypes are different. For example, several FGFR2 mutations associated with Crouzon or Pfeiffer phenotypes occur at the position equivalent to constitutively activating mutations associated with short limb skeletal dysplasias [60].

FGFR3 Mutations

FGFR3 p.Pro250Arg mutation causes Muenke syndrome, the most common syndromic form of craniosynostosis [60]. Muenke syndrome expressivity is extremely variable and nonpenetrance has been reported in some families. It is estimated that about 30% of children with coronal synostosis and 6–8% of all craniosynostosis patients have this mutation [71, 92].

A recurrent mutation in the transmembrane region of FGFR3, p.Ala391Glu, accounts for a form of Crouzon syndrome that associates craniosynostosis and *acanthosis nigricans* also called crouzonodermoskeletal syndrome [60].

FGFR3 mutations are also associated with four forms of short-limbed bone dysplasias. Of these, only thanatophoric dysplasias (types I and II) and rarely hypochondroplasia are associated with craniosynostosis. It is interesting that the penetrance of craniosynostosis varies from approximately 28% in thanatophoric dysplasia type I cases, with the p.Arg248Cys and p.Tyr373Cys

mutations, to 93% in thanatophoric dysplasias type II patients, carriers of the p.Lys650Glu mutation [60].

Twist Homolog Drosophila 1 (TWIST1)

Gene, Protein Structure and Functions

TWIST1, which encodes a basic helix-loop-helix transcription factor (bHLH), consists of a first exon with a translation start site (ATG) followed by an uninterrupted open reading frame of 606 nucleotides that encodes 202 amino acid residues (GenBank Accession No. U80998) and a second untranslated exon with two potential polyadenylation signals that are 65 and 415 bp away from the 5' end of exon 2.

TWIST1 protein is characterized by two highly conserved regions: a DNA-binding domain, which consists mostly of basic amino acid residues and the bHLH motif, which consists of a short alpha helix (helix I) connected by a loop to a second, longer alpha helix (helix II). The loop region is essential for the protein tertiary structure and the correct functionality of the two alpha helices. The HLH region is necessary and sufficient for protein dimerization, and dimerization is needed prior to DNA binding [100]. The second HLH protein can be the same (creating a homodimer) or different (creating a heterodimer). The repertoire of proteins that dimerizes with *Twist1* is not fully known, but it seems that the type of heterodimer formed is critical for determining the specificity of downstream target genes [101].

TWIST1 contains additional functional motifs. One of these (amino acids 30–64 in humans) binds to histone acetyltransferase p300 and can be involved in *Twist1* gene expression regulation through chromatin condensation [102]. Another motif is the 'Twist box' located at the C-terminal domain (amino acids 183–202 of the human protein), which interacts with the Runt-related factor DNA-binding domain, Runx2, and inhibits its function [103]. Nuclear localization sequences, other important functional domains, are located within and outside the HLH domain.

TWIST1 gene became a candidate for Saethre-Chotzen syndrome (SCS) after the gene was localized to chromosome 7p21, the region to which the SCS locus was previously mapped [104–107] and based on the observation that mice heterozygous for a *twist1*-null mutation exhibit subtle cranial and limb defects [108].

SCS, or acrocephalosyndactyly type III, is an autosomal dominantly inherited form of craniosynostosis. The classical clinical features are craniosynostosis, mainly involving the coronal sutures, low-set frontal hairline, facial asymmetry, ptosis of the eyelids, deviated nasal septum, brachydactily, partial soft tissue syndactily, especially of the second and third fingers, and various

skeletal anomalies, such as defects of the cervical and lumbar spine or radioulnar synostosis among others. Short stature has been documented in some instances. Patients may have hearing loss, most commonly due to conductive hearing impairment as a consequence of abnormal configuration of the nasopharynx, cleft palate or both. Penetrance is high, but it is not complete. There is a wide spectrum of clinical variability, and mild cases can often be misdiagnosed. A better characterization of the clinical spectrum of variability is being established since the identification of *TWIST1* as the causative gene.

TWIST1 Gene Mutations

Characterization and Distribution along the Gene

Although chromosomal structural alterations are not rare causative mechanisms of SCS, at least 97 different disease-causing mutations in the coding region of the *TWIST1* gene have been described among 153 patients worldwide mostly with SCS phenotype [109–115] (table 3). To date, no splice site and intronic mutations or changes within the promoter or in the second nontranslated exon have been reported. Among the mutations in the coding region of *TWIST1*, about 60% of are nucleotide substitutions leading to either missense (31%) or nonsense mutations (29%). The remaining 40% are deletions, duplications or insertions of less than 30 nucleotides, which will be referred to as small rearrangements (table 3). Two distinct mutations on the same allele have been reported in two unrelated patients.

The majority of the mutations (63/97; 65%) are located within the bHLH motif. The functional importance of the N- and C-terminal domains has also been shown by the identification of a few rare missense and in-frame mutations outside the bHLH motif [115]. Except for a few mutations, most of them are private with no apparent mutational hot spot. It is of note, however, that mutations involving the nucleotide at position c.309 (which encode the amino acid residue Tyr in the 5' DNA-binding domain) account for 8% of all mutations or 13% of the nucleotide substitutions (table 3). There is also an apparent excess of duplications starting at nucleotides 416–420 (encoding for amino acid residues in the loop region) and unequal crossovers due to repeated sequences in this region have been suggested as the causative mechanism [115].

Large deletions including the *TWIST1* gene account for at least 10% of the SCS cases. These deletions were originally identified by fluorescence in situ hybridization or Southern blot analysis [115], and their detection has been greatly facilitated with the use of the multiplex ligation-dependent probe amplification method. The extension of the deletion varies from a relatively small size encompassing mainly the *TWIST1* gene up to a few megabases.

Table 3. *TWIST1* mutations in patients with SCS and related phenotypes

Nucleotide change ^a ,	Consequence at the protein level	Functional domain	n	Reference No. ^c
c.7C>T	Gln3X	5' DNA binding	2	
c.61G>T	Glu21X	5' DNA binding	1	
c.82C>T	Gln28X	5' DNA binding	1	
c.106G>T	Gly36X	5' DNA binding	3	
c.108delA	Gly36GlyfsX88	5' DNA binding	1	
c.115C>G	p.Arg39Gly	NLS	1	
c.127_137del11	Arg43fsX233	5' DNA binding	1	
c.128_138del 11	Arg44fsX233	5' DNA binding	1	
c.181G>T	p.Gly61X	5' DNA binding	1	
c.193G>T	p.Glu65X	5' DNA binding	4	
c.211C>T	p.Gln71X	5' DNA binding	2	110
c.230delA;c.232T>C	Lys77fsX124	5' DNA binding	1	
c.263delG	Gly88fsX124	5' DNA binding	1	111
c.272_273ins 10	Ser93fsX292	5' DNA binding	1	
c.276_277dup21	Gly92_S93insGAGGGGG	5' DNA binding	2	
c.283delAinsCG	Ser95fsX237	5' DNA binding	1	
c.308_309insA	Tyr103X	5' DNA binding	2	
c.309C>A/G	Tyr103X	5' DNA binding	13	
c.309delC	Tyr103X	5' DNA binding	2	
c.310G>T	Glu104X	5' DNA binding	2	
c.326del17	p.Gln109fs	DNA binding	1	
Not referred	p.Gln109X	DNA binding	1	
c.336delG	p.Met112fsX12 ou	DNA binding	1	
c.340A>G	p.Asn114Asp	DNA binding	1	
Not referred	p.Asn114Ser	DNA binding	1	
c.346C>T	p.Arg116Trp	DNA binding	1	
Not referred	p.Arg116Gly	DNA binding	1	
c.348del17	p.Arg116fsX231	DNA binding	1	
c.352C>T	p.Arg118Cys	DNA binding	1	112
c.352_354del3	p.Arg118del	DNA binding	1	
c.353G>A	p.Arg118His	DNA binding	2	113
c.353G>C	p.Arg118Pro	DNA binding	1	
c.353_360del8	p.Arg118fsX234	DNA binding	1	
c.355C>T	p.Gln199X	DNA binding	2	
c.355delC	p.Gln119fsX124	DNA binding	1	
c.356A>C	p.Gln119Pro	DNA binding	1	
c.359G>C	p.Arg120Pro	DNA binding	1	
Not referred	p.Arg120Cys	DNA binding	1	
c.364C>T	p.Gln122X	helix 1	2	
c.368C>A	p.Ser123X	helix 1	3	
c.368C>G	p.Ser123Trp	helix 1	3	

Table 3. (continued)

Nucleotide change ^a ,	Consequence at the protein level	Functional domain	n	Reference No. ^c
c.376G>T	p.Glu126X	helix 1	4	
c.379G>A;c.398A>T	p.Ala127Thr/p.Lys133Ile	helix 1	1	
c.379_381dup	p.Ala127_Phe128insA	helix 1	1	
c.380C>A	p.Ala127Glu	helix 1	1	
c.384_385insC	p.Ala129fsX237	helix 1	1	
c.385G>C	p.Ala129Pro	helix 1	1	
c.385_405dup	p.Ala129_Ile135insAALRKII	helix 1	2	
c.392T>C	Leu131Pro	helix 1	1	
c.395G>C	Arg132Pro	helix 1	1	
c.397_417dup	p.Lys133_Pro139insKIIPITLP	helix1/loop	2	
c.402C>G	Ile134Met	helix 1	1	
c.405_406dup21	Ile135_Pro136insAALRKII	helix 1	2	
c.407C>T	Pro136Leu	helix 1	2	
Not referred	Pro136Leu	helix 1	1	
c.409A>C	Thr137Pro	helix 1	1	
c.415C>T	Pro139Ser	loop	1	
c.416C>A	Pro139His	loop	1	
c.416C>T	Pro139Leu	loop	1	
c.416_417dup21	Pro139_Ser140insKIIPITLP	loop	7	
c.417_418dup21	Pro139_Ser140ins KIIPITLP	loop	3	
c.418_419dup21	Ser140X	loop	1	
c.420_421dup21	Ser140_Asp141insIIPITLPS	loop	2	
c.421G>T	Asp141Tyr	loop	2	
c.422A>G	Asp141Gly	loop	1	
c.423_424ins25	Asp141_Lys142insDHPHAALGfsX297	loop	1	
c.428delT	p.Leu143ArgfsX76	loop	1	
c.430A>C	Ser144Arg	loop	2	
c.433A>G	Lys145Glu	loop	1	
c.433_455del23	Lys145fsX229	loop	1	
c.435G>C	Lys145Asn	loop	1	
c.442A>G	Thr148Ala	loop	1	
c.443C>A	THr148Asn	loop	1	
c.443C>G	Thr148Ser	loop	1	
c.443C>T	Thr148Ile	loop	1	
c.445C>T	Leu149Phe	loop	2	
c.454G>C	Ala152Pro	loop	1	
c.455C>T	Ala152Val	loop	1	
c.460A>G	Arg154Gly	helix 2	1	
c.460_461insA	Arg154fsX237	helix 2	1	
c.464_469del5	Tyr155X	helix2	1	
c.465C>A	Tyr155X	helix 2	1	

Table 3. (continued)

Nucleotide change ^a ,	Consequence at the protein level	Functional domain	n	Reference No. ^c
c.466A>G	Ile156Val	helix 2	1	
c.470A>T	Asp157Val	helix 2	1	
Not referred	Asp157Ala	helix 3	1	
c.472T>C	Phe158Leu	helix 2	2	
c.474C>G	Phe158Leu	helix 2	2	
c.475C>T	Leu159Phe	helix 2	1	
c.480C>G	Tyr160X	helix 2	1	
c.481C>T	Gln161X	helix 2	5	
c.481delC	Gln161fsX230	helix 2	1	
c.482_488del7	Gln161fsX228	helix 2	1	
c.485_488del4	Val162fsX229	helix 2	1	
c.487delC	Leu163fsX230	helix 2	1	
c.490C>T	Gln164X	helix 2	1	
c.495ins10	p.Ala165fs	helix 2	1	
c.541G>T	Glu181X	3' helix 2	1	
c.561C>G	Phe187Leu	TWIST box	1	
Deletion	entire gene	entire gene	20	

^aGenomic sequence based on GenBank accession No. U80998; mutations are referred to as originally reported.

^bMutation nomenclature according to Den Dunnen and Antonarakis [114].

^cAll the original reports of the mutations are cited in this table except for the ones included in Jabs (115).

The rearrangement (GGC)₅(CGC)(GGC)₅ at nucleotides 244–276 was initially identified only among affected patients. Family and functional studies have demonstrated that it is not pathogenic. This polyglycine tract variation does not seem to modulate the phenotype when in *cis* with a pathogenic mutation in the *TWIST1* gene, but its effect in *trans* with a pathogenic mutation has not yet been functionally or phenotypically addressed [115].

Polymorphisms within the *TWIST1* gene have also been identified. No functional or systematic studies have yet been performed for this type of mutations, but they do apparently not influence the phenotype.

Effect of the Mutations in the Protein

The phenotypes caused by complete heterozygous deletions of the *TWIST1* gene in some SCS patients and of the *Twist1* null heterozygous (*Twist*^{+/-}) mouse suggested that haploinsufficiency is the most likely disease-causing mechanism [116–118]. This assumption has been further reinforced by the

observation that all pathogenic mutations so far identified in SCS patients lead to a similar phenotype [115–117].

Different mutational mechanisms in *TWIST1* lead to haploinsufficiency in SCS. Nonsense mutations upstream or within the bHLH motif cause the synthesis of truncated proteins which are rapidly degraded, while missense mutations involving helix I or II regions create proteins that fail to heterodimerize and become abnormally located in the cytoplasm [119]. On the other hand, missense mutations in the loop-helix II junction region of the bHLH motif can lead to deficiency in protein-DNA interactions, while mutations in the middle of the loop seem to reduce heterodimerization with E12 protein and partial mislocalization of the protein [115]. Mutations in the *TWIST1* box lead to abnormal interaction with RUNX2 while a naturally occurring mutation in one of the NLS domain leads to nuclear mislocalization of Twist protein [110, 111]. Therefore, alterations in protein stability, dimerization deficiency, altered DNA-binding capacity or abnormal nuclear location can lead to *TWIST* loss of function.

Characterization of the breakpoints of some cytogenetically balanced translocations in SCS patients revealed that the coding region of *TWIST1* gene is preserved. Therefore, it is possible that the translocation breakpoints disrupt an important regulatory sequence of *TWIST1* or a second gene on 7p. Alternatively, the disease in these cases is caused by a positional effect on *TWIST1* expression [115, 120].

Phenotype and Genotype Correlations

Large pedigrees segregating nonsense mutations or a deletion of the *TWIST1* gene with the disease have exemplified the great clinical intrafamilial variability of the syndrome, including low penetrance for craniosynostosis in one of these genealogies [115]. A remarkable interfamilial clinical variability in SCS has also been well documented [60, 115]. The molecular mechanisms that cause the extreme variation in the clinical outcome of the disease are unknown, and there is no evidence of a correlation between the phenotype and the nature or location of a specific point or small rearrangement mutations within the *TWIST1* gene. Physical findings in patients with large gene deletions also do not differ from those with small or point mutations. Although intellectual deficits are rarely seen in patients with point mutations, they are often found in those with deletions encompassing the gene, thus suggesting a correlation between *TWIST1* gene deletions and cognitive function. It is still unclear whether the degree of mental retardation in patients with a complete deletion of the *TWIST1* gene is related to the size and location of the molecular defect [115, 121]. A more systematic study including delineation of the breakpoints and a better definition of the cognitive deficit in a larger number of patients is necessary to draw final conclusions.

The expression level of TWIST1 protein and of those that dimerize with it is critical for the correct function of TWIST1. We could thus speculate that variation on the availability of the counterpart proteins that dimerize with Twist1, which can be dependent on genetic or environmental factors, may play a role in the determination of the expressivity of the disease.

Despite the lack of genotype-phenotype correlation for physical alterations, analysis of the *TWIST1* gene in a large set of craniosynostotic patients has contributed to a better delineation of the syndrome. Bifid halluces and unilateral radial aplasia are now part of the clinical spectrum of variability of SCS as it was shown that patients diagnosed with Robinow-Sorauf and Baller-Gerold syndromes carry mutations in *TWIST1* [115]. In contrast, very mild patients, non-syndromic or in whom ptosis was the main clinical feature, have also been found to carry mutations in *TWIST1* [110, 115]. Therefore, the spectrum of clinical variability ranges from nonsyndromic or only ptosis to the full SCS phenotype, associated or not with bifid halluces and unilateral radial aplasia.

Some patients originally classified as SCS were found to have the p.Pro250Arg mutation at FGFR3 [109, 115], which is associated with Muenke syndrome. Therefore, it has been recommended that SCS patients negative for *TWIST1* mutations should be tested for this FGFR3 mutation. However, Kress et al. [110] argue that it is possible to clinically distinguish patients with SCS or Muenke syndrome. Based on the phenotype analysis of a large cohort of patients with *TWIST1* mutations or the p.Pro250Arg mutation in FGFR3, they suggested that low-set frontal hairline, gross ptosis of the eyelids, subnormal ear length, dilated parietal foramina, interdigital webbing, hallux valgus or broad great toe with bifid distal phalanx were significantly more prominent in patients with *TWIST1* mutations. In addition, intercranial hypertension as a consequence of early progressive multisutural fusion was a significant problem in SCS only, while mental delay and sensorineural hearing loss were associated with Muenke syndrome.

Several reports have shown that a few patients clinically classified as SCS do not harbor mutations either in the *TWIST1* gene or in the *FGFR* genes, suggesting genetic heterogeneity for the syndrome or a different mutational mechanism in these genes. No mutations have been found in potential candidate genes that are components of the same developmental pathway, including *SNAIL*, *SLUG* and *DERMO1* [109, 115].

Ephrin-B1 (EFNB1)

Gene and Protein Structure and Functions

Ephrins are one of the largest classes of membrane-bound ligands for Eph family receptor tyrosine kinases, which regulate cell adhesion and repulsion

responses that guide the migration of cells and axons along specific pathways during animal development. The ephrins and Eph proteins are also known to have an important function to avoid mixing of cells across boundaries in embryo development. Eph/ephrin interactions lead to the generation of a bidirectional signal, in which both the Eph receptors and the ephrins activate downstream signaling cascades simultaneously. Eight ephrins belonging to two classes have been characterized: class A (EFNA1–A5) which are linked to the cell membrane by a glycosylphosphatidylinositol anchor, and class B (EFNB1–B3) which are transmembrane proteins with intracellular region containing multiple tyrosine residues and a PDZ domain. Tyrosine phosphorylation and binding of PDZ-containing proteins are required for the function of transmembrane ephrins. The Eph receptors, 14 in number, are divided into EphA and EphB receptors, depending on their preferential affinity to ephrin-A or ephrin-B proteins [122–124].

Through linkage and positional candidate gene analysis it was demonstrated that mutations in one of the ephrin B genes, *EFNB1*, mapped at Xq13, cause a syndromic form of craniosynostosis, craniofrontonasal dysplasia [125, 126]. *EFNB1* contains 5 exons and encodes a protein of 346 aa.

Craniofrontonasal syndrome is an X-linked developmental disorder that shows greater severity in heterozygous females than in hemizygous males. Females present severe hypertelorism, coronal craniosynostosis either unilaterally or bilaterally, craniofacial asymmetry, frontal bossing, downslanting palpebral fissures, broad bifid nose, low posterior hairline with an anterior widow's peak, frizzy hair, and occasionally cleft lip or palate. Common extracranial features are sloping shoulders with dysplastic clavicles, mild cutaneous syndactyly, and characteristic longitudinal splitting of the nails, diaphragmatic hernia, and agenesis of corpus callosum. Males are rarely reported and paradoxically have a much milder phenotype, which includes hypertelorism and possibly cleft lip and/or palate [1, 125, 126]. Congenital diaphragmatic hernia can also be part of the phenotype in males, but its penetrance is still unknown [127].

EFNB1 Gene Mutations

Characterization and Distribution along the Gene

More than 70 different mutations distributed along the *EFNB1* gene have already been associated with craniofrontonasal syndrome; among these, 71 were intragenic and 3 were partial or complete gene deletions [125–130] (table 4). The intragenic mutations comprised 46 single-nucleotide and 1 double-nucleotide substitutions leading to missense or nonsense codons, splicing mutations or

Table 4. *EFNB1* mutations associated with craniofrontonasal dysplasia

Nucleotide change ^a	Exon (intron)	Consequence at mRNA or protein	Functional domain ^b	Cases n	Reference No.
4_4del	1	—	signal peptide	1	128
c.1A>G	1	p.Met1Val	signal peptide	1	125
c.30C>T ^c	1	p.Lys11SerfsX2	signal peptide	1	128
c.57G>A	1	p.Trp19X	signal peptide	1	125
c.80C>G	1	p.Pro27Arg	signal peptide	1	129
c.88_89delAA	1	p.Ala29fsX73	extracellular	1	129
c.109T>G	1	p.Trp37Gly	extracellular	2	128
c.110G>A	1	p.Trp37Gly	extracellular	1	128
c.123C>G	1	p.Asp41Lys	extracellular	1	128
c.146delG	2	p.Lys48fsX51	extracellular	1	129
c.151_153delGTG	2	p.Val51del	extracellular	1	127, 128
c.161C>T	2	p.Pro54Leu	extracellular	3	126, 128, 129
c.170_171GA>TT	2	p.Gly57Val	extracellular	1	128
c.185T>C	2	p.Ile62Thr	extracellular	1	125
c.191G>A	2	p.Cys64Tyr	extracellular	1	128
c.196delC	2	p.Arg66GluX93	extracellular	1	128
c.196C>T	2	p.Arg66X	extracellular	9	125, 128, 129
c.220G>T	2	p.Glu74X	extracellular	2	128, 129
c.229_232delAAGC	2	p.Tyr76fsX157	extracellular	1	129
c.233T>C	2	p.Leu78Pro	extracellular	1	128
c.246delG	2	p.Pro83fsX75	extracellular	1	125
c.258_261dupAGCT	2	p.Ala87fsX91	extracellular	1	129
c.265T>C	2	p.Cys89Arg	extracellular	1	128
c.266G>A	2	p.Cys89Tyr	extracellular	1	128
c.293T>C	2	p.Leu98Ser	extracellular	1	125
c.324_325insA	2	p.Ile108fsX131	extracellular	1	129
c.325delC	2	p.Ile108fsX158	extracellular	1	129
?T>C ^d	2	p.Thr111Ile	extracellular	1	126
c.339G>C	2	p.Lys113Asn	extracellular	1	128
c.344A>C	2	p.Gln115Pro	extracellular	1	128
c.346G>T	2	p.Glu116X	extracellular	1	129
c.355C>A	2	p.Pro119Thr	extracellular	1	125
c.355C>T	2	p.Pro119Ser	extracellular	1	129
c.355C>G	2	p.Pro119Ala	extracellular	1	128
c.356C>A	2	p.Pro119His	extracellular	2	125, 129
c.363C>A	2	p.Tyr121X	extracellular	1	128
c.368G>A	2	p.Gly123Asp	extracellular	1	128
c.377_384delTCAAGAAG	2	p.Glu125fsX128	extracellular	1	129
c.398delA	2	p.Tyr133SerfsX26	extracellular	1	128
c.406+1G>A	(2)	splice	extracellular	2	125, 128
c.407-1G>A	(2)	splice	extracellular	1	125
c.407-2A>G	(2)	splice	extracellular	1	129

Table 4. (continued)

Nucleotide change ^a	Exon (intron)	Consequence at mRNA or protein	Functional domain ^b	Cases n	Reference No.
c.407-2A>T	(2)	splice	extracellular	1	129
c.407C>T	3	p.Ser136Leu	extracellular	1	128
c.409A>G	3	p.Thr137Ala	extracellular	1	129
c.413C>T	3	p.Ser138Phe	extracellular	1	129
c.415delA	3	p.Ser138fsX158	extracellular	1	129
c.432delG	3	p.Leu145TrpfsX14	extracellular	2	127, 128
c.445G>T	3	p.Glu149X	extracellular	1	128
c.451G>A	3	p.Gly151Ser	extracellular	5	125, 128
c.452G>T	3	p.Gly151Val	extracellular	1	125
c.452G>A	3	p.Gly151Asp	extracellular	1	128
c.458G>A	3	p.Cys153Tyr	extracellular	1	129
c.458G>C	3	p.Cys153Ser	extracellular	2	125, 126
c.463A>C	3	p.Thr155Pro	extracellular	1	125
c.472A>G	3	p.Met158Val	extracellular	1	125
c.474G>T	3	p.Met158Ile	extracellular	1	125
c.496C>T	3	p.Gln166X	extracellular	1	128
c.500-2A>G	(3)	splicing	extracellular	1	128
c.546C>A	4	p.Ser182Arg	extracellular	1	129
c.550delG	4	p.Lys183fsX212	extracellular	1	129
c.564_565insT	4	p.Tyr189CysfsX10	extracellular	1	128
c.587delC	4	p.Pro196LeufsX17	extracellular	1	128
c.629-2A>G	(4)	splicing	extracellular	1	125
c.635_636delTG	5	p.Val212GluX19	extracellular	1	128
c.678_679insA	5	p.Ser226fsX231	extracellular	1	129
c.685_686insGG	5	p.Gly228fsX259	extracellular	1	129
c.685_686insG	5	p.Asp229GlyfsX31 ^e	extracellular	1	130
c.969delC	5	p.Gly322fsX391	cytoplasmic (potential)	1	129
c.986delA	5	p.Val328fsX391	cytoplasmic (potential)	1	129
c.993_994insCT	5	p.Gln332LeufsX61	cytoplasmic (potential)	1	128
Deletion	1–5	no protein	entire gene	2	128
Deletion	1–3	no protein	entire gene	1	128
Deletion exons	2–5	no protein	entire gene	1	129

^aNomenclature according to GenBank accession No. NM_004429.^bDomains according to <http://us.expasy.org>.^cMutation causes altered splicing.^dPublished numbering incompatible with exon 2 sequence.^eOriginally referred to as p.Gly230fsX.

alteration of the initiation codon; the remaining ones were frameshifting deletions, insertions or duplications and one in-frame deletion mutations. Of the 48 single nucleotide substitutions identified among 66 unrelated patients, only 2 mutations were more highly recurrent, respectively, at nucleotides c.196C (9/66) and c.451G (5/66); however, there is no evidence of a mutational hot spot along the gene. Nucleotide substitutions are located in exons 1–4 of the gene (encoding for the signal peptide/extracellular domains) while nearly 50% of the frameshift deletions/insertions occurred within the last two exons of the *EFNB1* gene (which encode part of the transmembrane and cytoplasmic domains).

Effect of the Mutation on the Protein

Missense mutations change highly conserved amino acid residues across species in the extracellular ephrin domain and are expected to disrupt protein folding or interaction sites for ephrin-B1 interacting partners (such as ephrin-B2 and EphB2), and thus correspond to loss-of-function mutations. The frameshift, nonsense and splice site mutations identified in exons 1–4 of the *EFNB1* gene generate premature termination codons that most likely elicit nonsense-mediated mRNA decay and therefore correspond to null mutants. Whether this also applies to the frameshift mutations in exon 5 is not quite clear, because premature termination occurs in the last exon of the gene. This may allow the synthesis of a truncated, soluble ephrin-B1 that could act in a dominant-negative fashion provided that it is properly processed and expressed as a stable polypeptide. Other frameshift mutations at the end of exon 5 change the reading frame and lead to the addition of several amino acids at the carboxyterminal part of ephrin-B1. These mutations are predicted to alter the structure of the cytoplasmic tail and disrupt the intracellular binding sites for Grb4 and PDZ-effector proteins, which are involved in ephrin-B1 reverse signaling. These C-terminal frameshifts may also impair bidirectional endocytosis of ephrin-B1 and EphB complexes, a mechanism that appears to regulate Eph-ephrin contact-mediated repulsion [125–129].

Genotype-Phenotype Correlations

Mutations in *EFNB1* have been found in the great majority of females (~95%) diagnosed with craniofrontonasal syndrome, thus suggesting genetic heterogeneity or a still unknown mutational mechanism in this gene in less than 10% of the cases [129].

There is great intrafamilial and interfamilial clinical variability among craniofrontonasal female subjects, including the extent of craniosynostosis and the occurrence of additional clinical features. No genotype-phenotype correlation is apparent in any of the studies, which implies that missense or frameshift changes cause a comparable disturbance of ephrin-B1 [125–129]. This X-linked disease has been a paradox in human genetics, as heterozygous female carriers

of the mutation always present a more severe phenotype than hemizygous males. In addition, there is a significant excess of females.

It has been proposed that the phenotypic discrepancies between the sexes are due to cellular interference, a process associated with X inactivation. That is, in heterozygous females, there is a mosaic of cells expressing and not expressing ephrin-B1, which might interfere in the establishment of tissue boundaries during embryogenesis. On the other hand, as males will only have cells without *EFNB1*, no disruption in cell boundaries will occur and ligand/receptor promiscuity may explain the mild or absent manifestation of *EFNB1* mutations in hemizygotes [125, 126]. This hypothesis has been supported by the observation that mice harboring *Efnb1* null mutation show a similar paradoxical pattern of phenotypic severity, with heterozygous females consistently more severely affected than hemizygous males. In the heterozygous female mice, abnormal sorting of cells into ephrin-B1-expressing and ephrin-B1-nonexpressing patches was shown to correlate with the X-inactivation status of *Efnb1* [131]. However, this hypothesis does not explain the occurrence of congenital diaphragmatic hernia, as this malformation is present both in males and females with mutations in *EFNB1*. It is possible that ephrin-B1 does not have a nonredundant role in the development of the diaphragm [127].

The low proportion of affected males has been shown to be associated with the origin of the mutation, as 92% of the de novo mutations occur in the paternal germinative cells [128]. It is also of note that somatic mosaicism is quite high, accounting for 18.5% of the cases. These data have important implications for genetic counseling.

Final Considerations

The growth of the skull, which involves ossification and growth of the cranial plates and their fusion along the calvarial sutures, is very well coordinated with the growth of the developing brain and it might reflect an important evolutionary process. Identification of the molecular pathways involved in this developmental process will not only provide insights into the understanding of this process but will also have an important impact on diagnosis and genetic counseling.

Syndromic craniosynostosis is etiologically very heterogeneous and the involvement of several genes in the control of suture development is suggested by the diverse number of chromosomal abnormalities. This group of conditions is possibly caused by haploinsufficiency or gain-of-function mechanisms represented, respectively, by deletions and duplications. The identification of the genes within these chromosomal rearrangements involved with suture development remains a challenge.

The genes so far identified that, when mutated, unequivocally cause craniosynostosis belong to four functional groups: regulatory molecules at the DNA level: TWIST1 and MSX2, tyrosine-kinase receptors: FGFR1–3, ligand receptors: EFNB1 and EFNA4, and intracellular trafficking of membrane-associated protein: RAB23. RAB23 is the only one associated with an autosomal recessive condition, while mutations in the others cause autosomal dominant disorders. Five of these genes are involved in the process of cell proliferation and ossification and they seem to belong to a common molecular pathway. Gain-of-function mutations represent the main molecular mechanism causing the disorders, but loss of function can also lead to the phenotype. The association of ephrin genes with craniosynostosis has shed new light on the understanding of suture development, as they provided evidence that craniosynostosis, at least when involving coronal sutures, can be the result of a defect in the boundary formation between cellular compartments during suture formation. Twist, Msx2 and possibly FGFR2 may also be involved in this process, at least in tissues where these genes are coexpressed.

Although the number of patients with mutations in FBN1, TGFBR1 and 2 is still small and the penetrance of craniosynostosis is low, they provide insights into the importance of the extracellular matrix components and their signaling in suture development.

This is a very exciting field in human genetics and the molecular analysis in patients with craniosynostosis has made a major and significant contribution to the understanding of this complex mechanisms.

References

- 1 Cohen MM Jr: ■■■■; in *Craniosynostosis: Diagnosis, Evaluation and Management*, ed 2. New York, Oxford University Press, 2000.
- 2 Lajeunie E, Le Merrer M, Bonaiti-Pellie C, Marchac D, Renier D: Genetic study of nonsyndromic coronal craniosynostosis. *Am J Med Genet* 1995;55:500–504.
- 3 Lajeunie E, Le Merrer M, Bonaiti-Pellie C, Marchac D, Renier D: Genetic study of scaphocephaly. *Am J Med Genet* 1996;62:282–285.
- 4 Lajeunie E, Le Merrer M, Marchac D, Renier D: Syndromal and nonsyndromal primary trigonocephaly: analysis of a series of 237 patients. *Am J Med Genet* 1998;2:211–215.
- 5 Hennekam RCM, Van den Boogaard MJ: Autosomal dominant craniosynostosis of the sutura metopica. *Clin Genet* 1990;38:374–377.
- 6 Merrill AE, Bochukova EG, Brugger SM, Ishii M, Pilz DT, Wall SA, Lyons KM, Wilkie AO, Maxson RE Jr: Cell mixing at a neural crest-mesoderm boundary and deficient ephrin-Eph signaling in the pathogenesis of craniosynostosis. *Hum Mol Genet* 2006;15:1319–1328.
- 7 Jenkins D, Seelow D, Jehee FS, Perlyn CA, Alonso LG, Bueno DF, Donnai D, Josifiova D, Mathijssen IMJ, Morton JEV, Ørstavik KH, Sweeney E, Wall SA, Marsh JL, Nürnberg P, Passos-Bueno MR, Wilkie AOM: *RAB23* mutations in Carpenter syndrome imply an unexpected role for hedgehog signaling in cranial-suture development and obesity. *Am J Hum Genet* 2007;80:1162–1170.
- 8 Sood S, Eldadah ZA, Krause WL, McIntosh I, Dietz HC: Mutation in fibrillin-1 and the Marfanoid-craniosynostosis (Shprintzen-Goldberg) syndrome. *Nat Genet* 1996;12:209–211.

- 9 Flück CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Verge CF, Jabs EW, Mendonca BB, Fujieda K, Miller WL: Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. *Nat Genet* 2004;36:228–230.
- 10 Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, Meyers J, Leitch CC, Katsanis N, Sharifi N, Xu FL, Myers LA, Spevak PJ, Cameron DE, De Backer J, Hellemans J, Chen Y, Davis EC, Webb CL, Kress W, Coucke P, Rifkin DB, De Paepe AM, Dietz HC: A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* 2005;37:275–281.
- 11 Cohen MM Jr: Sutural biology and the correlates of craniosynostosis. *Am J Med Genet* 1993;47:581–616.
- 12 Gajecka M, Yu W, Ballif BC, Glotzbach CD, Bailey KA, Shaw CA, Kashork CD, Heilstedt HA, Ansel DA, Theisen A, Rice R, Rice DP, Shaffer LG: Delineation of mechanisms and regions of dosage imbalance in complex rearrangements of 1p36 leads to a putative gene for regulation of cranial suture closure. *Eur J Hum Genet* 2005;13:139–149.
- 13 Van Buggenhout G, Van Ravenswaaij-Arts C, McMaas N, Thoelen R, Vogels A, Smeets D, Salden I, Matthijs G, Fryns JP, Vermeesch JR: The del(2)(q32.2q33) deletion syndrome defined by clinical and molecular characterization of four patients. *Eur J Med Genet* 2005;48:276–289.
- 14 Schwyzer U, Binkert F, Caflisch U, Baumgartner B, Schinzel A: Terminal deletion of the short arm of chromosome 3, del(3pter-p25): a recognizable syndrome. *Helv Paediatr Acta* 1987;42:309–315.
- 15 Tipton RE, Berns JS, Johnson WE, Wilroy RS Jr, Summitt RL: Duplication 6q syndrome. *Am J Med Genet* 1979;3:325–330.
- 16 Van Buggenhout G, Maas NM, Fryns JP, Vermeesch JR: A dysmorphic boy with 4qter deletion and 4q32.3–34.3 duplication: clinical, cytogenetic, and molecular findings. *Am J Med Genet* 2004;131:186–189.
- 17 Zneimer SM, Cotter PD, Stewart SD: Telomere-telomere (end to end) fusion of chromosomes 7 and 22 with an interstitial deletion of chromosome 7p11.2→p15.1: phenotypic consequences and possible mechanisms. *Clin Genet* 2000;58:129–133.
- 18 Hinkel GK, Tolkendorf E, Bergan J: 7p-deletion syndrome (in German). *Monatsschr Kinderheilkd* 1988;136:824–827.
- 19 Fryns JP, Haspelslagh M, Agneessens A, van den Berghe H: De novo partial 2q3 trisomy/distal 7p22 monosomy in a malformed newborn with 7p deletion phenotype and craniosynostosis. *Ann Genet* 1985;28:45–48.
- 20 Morimoto M, An B, Ogami A, Shin N, Sugino Y, Sawai Y, Usuku T, Tanaka M, Hirai K, Nishimura A, Hasegawa K, Sugimoto T: Infantile spasms in a patient with williams syndrome and craniosynostosis. *Epilepsia* 2003;44:1459–1462.
- 21 Fryburg JS, Golden WL: Interstitial deletion of 8q13.3→22.1 associated with craniosynostosis. *Am J Med Genet* 1993;45:638–641.
- 22 Eshel G, Lahat E, Reish O, Barr J: Neurodevelopmental and behavioral abnormalities associated with deletion of chromosome 9p. *J Child Neurol* 2002;17:50–51.
- 23 Schimmenti LA, Higgins RR, Mendelsohn NJ, Casey TM, Steinberger J, Mammel MC, Wiesner GL: Monosomy 9p24→pter and trisomy 5q31→qter: case report and review of two cases. *Am J Med Genet* 1995;57:52–56.
- 24 Redon R, Baujat G, Sanlaville D, Le Merrer M, Vekemans M, Munnich A, Carter NP, Cormier-Daire V, Colleaux L: Interstitial 9q22.3 microdeletion: clinical and molecular characterisation of a newly recognised overgrowth syndrome. *Eur J Hum Genet* 2006;14:759–767.
- 25 Yatsenko SA, Cheung SW, Scott DA, Nowaczyk MJ, Tarnopolsky M, Naidu S, Bibat G, Patel A, Leroy JG, Scaglia F, Stankiewicz P, Lupski JR: Deletion 9q34.3 syndrome: genotype-phenotype correlations and an extended deletion in a patient with features of Opitz C trigonocephaly. *J Med Genet* 2005;42:328–335.
- 26 Francke U, Kernahan C, Bradshaw C: Del (10)p autosomal deletion syndrome: clinical, cytogenetic and gene marker studies. *Humangenetik* 1975;26:343–351.
- 27 Lukusa T, Smeets E, Vermeesch JR, Fryns JP: Small terminal 10q26 deletion in a male patient with Noonan-like stigmata: diagnosis by cytogenetic and FISH analysis. *Genet Couns* 2002;13:417–425.

- 28 Masuno M, Asano J, Yasuda K, Kondo T, Orii T: Balanced complex rearrangement involving chromosomes 8, 9, and 12 in a normal mother, derivative chromosome 9 with recombinant chromosome 12 in her daughter with minor anomalies. *Am J Med Genet* 1993;45:65–67.
- 29 Lemyre E, Lemieux N, Decarie JC, Lambert M: Del(14)(q22.1q23.2) in a patient with anophthalmia and pituitary hypoplasia. *Am J Med Genet* 1998;77:162–165.
- 30 Shur N, Cowan J, Wheeler PG: Craniosynostosis and congenital heart anomalies associated with a maternal deletion of 15q15–22.1. *Am J Med Genet A* 2003;120:542–546.
- 31 Fukushima Y, Wakui K, Nishida T, Nishimoto H: Craniosynostosis in an infant with an interstitial deletion of 15q [46,XY,del(15)(q15q22.1)]. *Am J Med Genet* 1990;36:209–213.
- 32 Thomas JA, Manchester DK, Prescott KE, Milner R, McGavran L, Cohen MM Jr: Hunter-McAlpine craniosynostosis phenotype associated with skeletal anomalies and interstitial deletion of chromosome 17q. *Am J Med Genet* 1996;62:372–375.
- 33 McDonald-McGinn DM, Gripp KW, Kirschner RE, Maisenbacher MK, Hustead V, Schauer GM, Keppler-Noreuil KM, Ciprero KL, Pasquariello P Jr, LaRossa D, Bartlett SP, Whitaker LA, Zackai EH: Craniosynostosis: another feature of the 22q11.2 deletion syndrome. *Am J Med Genet A* 2005;136:358–362.
- 34 Lucas J, Faivre J, Le Mee F, Hubert S, Pluquailec K, Picard F: De novo interstitial deletion of the long arm of chromosome 2: 46,XXX,del(2)(q14q21), associated with premature craniosynostosis (in French). *Ann Genet* 1987;30:33–38.
- 35 Gagnon JA, Richer CL, Lemieux N, Gauthier-Chouinard M: De novo appearance of a partial trisomy 1q in mosaic due to a 1;9 translocation (in French). *Ann Genet* 1984;27:33–37.
- 36 de Brasi D, Perone L, di Micco P, Andria G, Sebastio G, Iaccarino E, Pinto L, Aliberti F: Cloverleaf skull anomaly and de novo trisomy 4p. *J Med Genet* 1999;36:422–424.
- 37 Breslau-Siderius EJ, Wijnen JT, Dauwerse JG, de Pater JM, Beemer FA, Khan PM: Paternal duplication of chromosome 5q11.2–5q14 in a male born with craniostenosis, ear tags, kidney dysplasia and several other anomalies. *Hum Genet* 1993;92:481–485.
- 38 Shiihara T, Kato M, Kimura T, Hayasaka K, Yamamori S, Ogata T: Craniosynostosis with extra copy of MSX2 in a patient with partial 5q-trisomy. *Am J Med Genet A* 2004;128:214–216.
- 39 Odell JM, Siebert JR, Bradley C, Salk D: Duplication 7p in a family with t(7;11): association with anomalies of the anterior cranial base. *Am J Med Genet* 1987;27:687–692.
- 40 Seghezzi L, Maraschio P, Bozzola M, Maserati E, Tupler R, Marchi A, Tiepolo L: Ring chromosome 9 with a 9p22.3–p24.3 duplication. *Eur J Pediatr* 1999;158:791–793.
- 41 Yelavarthi KK, Zunich J: Familial interstitial duplication of 11q; partial trisomy (11)(q13.5q21). *Am J Med Genet* 2004;126A:423–426.
- 42 Babovic-Vuksanovic D, Westman JA, Jalal SM, Lindor NM: Clinical characteristics associated with dup17(q24q25.1) in a mosaic mother and two non-mosaic daughters. *Clin Dysmorphol* 1998;7:171–176.
- 43 Czako M, Riegel M, Morava E, Bajnoczky K, Kosztolanyi G: Opitz 'C' trigonocephaly-like syndrome in a patient with terminal deletion of 2p and partial duplication of 17q. *Am J Med Genet A* 2004;131:310–312.
- 44 Jehee FS, Rosenberg C, Krepschi-Santos AC, Kok F, Knijnenburg J, Froyen G, Vianna-Morgante AM, Opitz JM, Passos-Bueno MR: An Xq22.3 duplication detected by comparative genomic hybridization microarray (Array-CGH) defines a new locus (FGS5) for FG syndrome. *Am J Med Genet A* 2005;139:221–226.
- 45 Ward J, Vieto E, Lee D, Arosemena G: Acrocephalopolysyndactyly, pentalogy of Fallot, and hypoacusis in a patient with a de novo reciprocal translocation involving the short arm of chromosome 1 and the long arm of chromosome 18:46,XX,t(1;18)(p31;q11). *J Med Genet* 1993;30:438–439.
- 46 Hordnes K, Engebretsen LF, Knudtzon J: De novo balanced 5;21 translocation in a child with acrobachycephaly, ventriculomegaly, pulmonary stenosis, ectopic anus and mental retardation. *Clin Genet* 1995;48:321–323.
- 47 Kaminker CP, Dain L, Lamas MA, Sanchez JM: Mosaic trisomy 9 syndrome with unusual phenotype. *Am J Med Genet* 1985;22:237–241.
- 48 Denyer W, Plamer C: Closed metopic suture with trigonocephaly in Down's syndrome. *Neurology* 1965;15:756–760.

- 49 Jehee FS, Bertola DR, Yelavarthi KK, Krepischi-Santos ACV, Vermeesch JR, Passos-Bueno MR: An 11q11-q13.3 duplication, including *FGF3* and *FGF4* genes, in a patient with syndromic multiple craniosynostosis. *Am J Med Genet A*, in press.
- 50 Brewer C, Holloway S, Zawalnski P, Schinzel A, FitzPatrick D: A chromosomal deletion map of human malformations. *Am J Hum Genet* 1998;63:1153–1159.
- 51 Brewer C, Holloway S, Zawalnski P, Schinzel A, FitzPatrick D: A chromosomal duplication map of malformations: regions of suspected haplo- and triplolethality – and tolerance of segmental aneuploidy – in humans. *Am J Hum Genet* 1999;64:1702–1708.
- 52 Huret JL, Leonard C, Forestier B, Rethoré MO, Lejeune J: Eleven new cases of del(9p) and features from 80 cases. *J Med Genet* 1988;25:741–749.
- 53 de Pater JM, Ippel PF, Bijlsma JB, Van Nieuwenhuizen O: Interstitial deletion 11q case report and review of the literature. *Genet Couns* 1997;4:335–339.
- 54 Christ LA, Crowe CA, Micale MA, Conroy J, Schwartz S: Chromosome breakage hotspots and delineation of the critical region for the 9p-deletion syndrome. *Am J Hum Genet* 1999;65:1387–1395.
- 55 Leegde B, Kerstjens-Frederix WS, Deelstra K, Begeer JH, Van Essen AJ: 11q-syndrome: three cases and a review of the literature. *Genet Couns* 1999;3:305–313.
- 56 Ornitz DM: FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays* 2000;22:108–112.
- 57 Givol D, Eswarakumar VP, Lonai P: The fibroblast growth factor signaling pathway; in Epstein CJ, Erickson RP, Wynshaw-Boris A (eds): *Inborn Errors of Development*. Oxford, Oxford University Press, 2004, pp 367–379.
- 58 Eswarakumar VP, Lax I, Schlessinger J: Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* 2005;16:139–149.
- 59 Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G, Goldfarb M: Receptor specificity of the fibroblast growth factor family. *J Biol Chem* 1996;271:15292–15297.
- 60 Cohen MM: FGFs/FGFRs and association disorders; in Epstein CJ, Erickson RP, Wynshaw-Boris A (eds): *Inborn Errors of Development*. Oxford, Oxford University Press, 2004, pp 380–400.
- 61 Roscioli T, Flanagan S, Kumar P, Masel J, Gattas M, Hyland VJ, Glass IA: Clinical findings in a patient with FGFR1 P252R mutation and comparison with the literature. *Am J Med Genet* 2000;93:228.
- 62 White KE, Cabral JM, Davis SI, Fishburn T, Evans WE, Ichikawa S, Fields J, Yu X, Shaw NJ, McLellan NJ, McKeown C, Fitzpatrick D, Yu K, Ornitz DM, Econs MJ: Mutations that cause osteoglophonic dysplasia define novel roles for FGFR1 in bone elongation. *Am J Hum Genet* 2005;76:361–367.
- 63 Wilkie AO: Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations. *Cytokine Growth Factor Rev* 2005;16:187–203.
- 64 Priolo M, Lerone M, Baffico M, Baldi M, Ravazzolo R, Cama A, Capra V, Silengo M: Pfeiffer syndrome type 2 associated with a single amino acid deletion in the FGFR2 gene. *Clin Genet* 2000;58:81–83.
- 65 Lapunzina P, Fernandez A, Sanchez Romero JM, Delicado A, Saenz de Pipaon M, Lopez Pajares I, Molano J: A novel insertion in the FGFR2 gene in a patient with Crouzon phenotype and sacro-coccygeal tail. *Birth Defects Res A Clin Mol Teratol* 2005;73:614.
- 66 Kress W, Collmann H, Büsse M, Halliger-Keller B, Mueller CR: Clustering of FGFR2 gene mutations in patients with Pfeiffer and Crouzon syndromes (FGFR2 associated craniosynostoses). *Cytogenet Cell Genet* 2000;91:1347.
- 67 Passos-Bueno MR, Sertie AL, Richieri-Costa A, Alonso LG, Zatz M, Alonso N, Brunoni D, Ribeiro SF: Description of a new mutation and characterization of FGFR1, FGFR2, and FGFR3 mutations among Brazilian patients with syndromic craniosynostoses. *Am J Med Genet* 1998;78:237–241.
- 68 Lajeunie E, Heuertz S, El Ghouzzi V, Martinovic J, Renier D, Le Merrer M, Bonaventure J: Mutation screening in patients with syndromic craniosynostoses indicates that a limited number of recurrent FGFR2 mutations accounts for severe forms of Pfeiffer syndrome. *Eur J Hum Genet* 2006;14:289–298.

- 69 Gorry MC, Preston RA, White GJ, Zhang Y, Singhal VK, Losken HW, Parker MG, Nwokoro NA, Post JC, Ehrlich GD: Crouzon syndrome: mutations in two spliceoforms of FGFR2 and a common point mutation shared with Jackson-Weiss syndrome. *Hum Mol Genet* 1995;4:1387–1390.
- 70 Freitas EC, Nascimento SR, de Mello MP, Gil-da-Silva-Lopes VL: Q289P mutation in FGFR2 gene causes Saethre-Chotzen syndrome: some considerations about familial heterogeneity. *Cleft Palate Craniofac J* 2006;43:142–147.
- 71 Wilkie AO, Bochukova EG, Hansen RM, Taylor IB, Rannan-Eliya SV, Byren JC, Wall SA, Ramos L, Venancio M, Hurst JA, O'Rourke AW, Williams LJ, Seller A, Lester T: Clinical dividends from the molecular genetic diagnosis of craniosynostosis. *Am J Med Genet A* 2006;140:2631–2639.
- 72 Tartaglia M, Di Rocco C, Lajeunie E, Valeri S, Velardi F, Battaglia PA: Jackson-Weiss syndrome: identification of two novel FGFR2 missense mutations shared with Crouzon and Pfeiffer craniosynostotic disorders. *Hum Genet* 1997;101:47–50.
- 73 Park WJ, Meyers GA, Li X, Theda C, Day D, Orlow SJ, Jones MC, Jabs EW: Novel FGFR2 mutations in Crouzon and Jackson-Weiss syndromes show allelic heterogeneity and phenotypic variability. *Hum Mol Genet* 1995;4:1229–1233.
- 74 Meyers GA, Day D, Goldberg R, Daentl DL, Przylepa KA, Abrams LJ, Graham JM Jr, Feingold M, Moeschler JB, Rawnsley E, Scott AF, Jabs EW: FGFR2 exon IIIa and IIIc mutations in Crouzon, Jackson-Weiss, and Pfeiffer syndromes: evidence for missense changes, insertions, and a deletion due to alternative RNA splicing. *Am J Hum Genet* 1996;58:491–498.
- 75 Pulley LJ, Reardon W, Wilkes D, Rutland P, Jones BM, Hayward R, Hall CM, Brueton L, Chun N, Lammer E, Malcolm S, Winter RM: Spectrum of craniosynostosis phenotypes associated with novel mutations at the fibroblast growth factor receptor 2 locus. *Eur J Hum Genet* 1996;4:283–291.
- 76 Okajima K, Robinson LK, Hart MA, Abuelo DN, Cowan LS, Hasegawa T, Maumenee IH, Jabs EW: Ocular anterior chamber dysgenesis in craniosynostosis syndromes with a fibroblast growth factor receptor 2 mutation. *Am J Med Genet* 1999;85:160–170.
- 77 Everett ET, Britto DA, Ward RE, Hartsfield JK Jr: A novel FGFR2 gene mutation in Crouzon syndrome associated with apparent nonpenetrance. *Cleft Palate Craniofac J* 1999;36:533–541.
- 78 de Ravel TJ, Taylor IB, Van Oostveldt AJ, Fryns JP, Wilkie AO: A further mutation of the FGFR2 tyrosine kinase domain in mild Crouzon syndrome. *Eur J Hum Genet* 2005;13:503–505.
- 79 McGillivray G, Savarirayan R, Cox TC, Stojkoski C, McNeil R, Bankier A, Bateman JF, Roscioli T, Gardner RJ, Lamande SR: Familial scaphocephaly syndrome caused by a novel mutation in the FGFR2 tyrosine kinase domain. *J Med Genet* 2005;42:656–662.
- 80 Zankl A, Jaeger G, Bonafe L, Boltshauser E, Superti-Furga A: Novel mutation in the tyrosine kinase domain of FGFR2 in a patient with Pfeiffer syndrome. *Am J Med Genet A* 2004;131:299–300.
- 81 Schindler S, Friedrich M, Wagener H, Lorenz B, Preising MN: Heterozygous P250L mutation of fibroblast growth factor receptor 3 in a case of isolated craniosynostosis. *J Med Genet* 2002;39:764–766.
- 82 Tavormina PL, Shiang R, Thompson LM, Zhu YZ, Wilkin DJ, Lachman RS, Wilcox WR, Rimoin DL, Cohn DH, Wasmuth JJ: Thanatophoric dysplasia (types I and II) caused by distinct mutations in fibroblast growth factor receptor 3. *Nat Genet* 1995;9:321–328.
- 83 Shiang R, Thompson LM, Zhu YZ, Church DM, Fielder TJ, Bocian M, Winokur ST, Wasmuth JJ: Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell* 1994;78:335–342.
- 84 Bellus GA, McIntosh I, Smith EA, Aylsworth AS, Kaitila I, Horton WA, Greenhaw GA, Hecht JT, Francomano CA: A recurrent mutation in the tyrosine kinase domain of fibroblast growth factor receptor 3 causes hypochondroplasia. *Nat Genet* 1995;10:357–359.
- 85 Tavormina PL, Bellus GA, Webster MK, Bamshad MJ, Fraley AE, McIntosh I, Szabo J, Jiang W, Jabs EW, Wilcox WR, Wasmuth JJ, Donoghue DJ, Thompson LM, Francomano CA: A novel skeletal dysplasia with developmental delay and acanthosis nigricans is caused by a Lys650Met mutation in the fibroblast growth factor receptor 3 gene. *Am J Hum Genet* 1999;64:722–731.
- 86 Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, Pecheux C, Le Tessier D, Cruaud C, Delpech M, Speleman F, Vermeulen S, Amalfitano A, Bachelot Y, Bouchard P, Cabrol S, Carel JC, Deleamarre-van de Waal H, Goulet-Salmon B, Kottler ML, Richard O, Sanchez-Franco F, Saura R, Young J,

- Petit C, Hardelin JP: Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33:463–465.
- 87 Rohmann E, Brunner HG, Kayserili H, et al: Mutations in different components of FGF signaling in LADD syndrome. *Nat Genet* 2006;38:414–417.
- 88 Toydemir RM, Brassington AE, Bayrak-Toydemir P, Krakowiak PA, Jorde LB, Whitby FG, Longo N, Viskochil DH, Carey JC, Bamshad MJ: A novel mutation in FGFR3 causes camptodactyly, tall stature, and hearing loss (CATSHL) syndrome. *Am J Hum Genet* 2006;79:935–941.
- 89 Moloney DM, Slaney SF, Oldridge M, Wall SA, Sahlin P, Stenman G, Wilkie AO: Exclusive paternal origin of new mutations in Apert syndrome. *Nat Genet* 1996;13:48–53.
- 90 Glaser RL, Jiang W, Boyadjiev SA, Tran AK, Zachary AA, Van Maldergem L, Johnson D, Walsh S, Oldridge M, Wall SA, Wilkie AO, Jabs EW: Paternal origin of FGFR2 mutations in sporadic cases of Crouzon syndrome and Pfeiffer syndrome. *Am J Hum Genet* 2000;66:768–777.
- 91 Kan SH, Elanko N, Johnson D, Cornejo-Roldan L, Cook J, Reich EW, Tomkins S, Verloes A, Twigg SR, Rannan-Eliya S, McDonald-McGinn DM, Zackai EH, Wall SA, Muenke M, Wilkie AO: Genomic screening of fibroblast growth factor receptor 2 reveals a wide spectrum of mutations in patients with syndromic craniosynostosis. *Am J Hum Genet* 2002;70:472–486.
- 92 Morriss-Kay GM, Wilkie AO: Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. *J Anat* 2005;207:637–653.
- 93 Wilkie AO, Patey SJ, Kan SH, van den Ouweland AM, Hamel BC: FGFs, their receptors, and human limb malformations: clinical and molecular correlations. *Am J Med Genet* 2002;112:266–278.
- 94 Slaney SF, Oldridge M, Hurst JA, Morriss-Kay GM, Hall CM, Poole MD, Wilkie AO: Differential effects of FGFR2 mutations on syndactyly and cleft palate in Apert syndrome. *Am J Hum Genet* 1996;58:923–932.
- 95 Anderson J, Burns HD, Enriquez-Harris P, Wilkie AO, Heath JK: Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. *Hum Mol Genet* 1998;7:1475–1483.
- 96 Yu K, Herr AB, Waksman G, Ornitz DM: Loss of fibroblast growth factor receptor 2 ligand-binding specificity in Apert syndrome. *Proc Natl Acad Sci USA* 2000;97:14536–14541.
- 97 Ibrahimi OA, Zhang F, Eliseenkova AV, Itoh N, Linhardt RJ, Mohammadi M: Biochemical analysis of pathogenic ligand-dependent FGFR2 mutations suggests distinct pathophysiological mechanisms for craniofacial and limb abnormalities. *Hum Mol Genet* 2004;13:2313–2324.
- 98 Ibrahimi OA, Zhang F, Eliseenkova AV, Linhardt RJ, Mohammadi M: Proline to arginine mutations in FGF receptors 1 and 3 result in Pfeiffer and Muenke craniosynostosis syndromes through enhancement of FGF binding affinity. *Hum Mol Genet* 2004;13:69–78.
- 99 Oldridge M, Zackai EH, McDonald-McGinn DM, Iseki S, Morriss-Kay GM, Twigg SR, Johnson D, Wall SA, Jiang W, Theda C, Jabs EW, Wilkie AO: De novo alu-element insertions in FGFR2 identify a distinct pathological basis for Apert syndrome. *Am J Hum Genet* 1999;64:446–461.
- 100 Murre C, Murre C, Bain G, van Dijk MA, Engel I, Furnari BA, Massari ME, Matthews JR, Quong MW, Rivera RR, Stuiver MH: Structure and function of helix-loop-helix proteins. *Biochim Biophys Acta* 1994;1218:129–135.
- 101 Connerney J, Andreeva V, Leshem Y, Muentener C, Mercado MA, Spicer DB: Twist1 dimer selection regulates cranial suture patterning and fusion. *Dev Dyn* 2006;235:1345–1357.
- 102 Hamamori Y, Sartorelli V, Ogryzko V, Puri PL, Wu HY, Wang JY, Nakatani Y, Kedes L: Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell* 1999;96:405–413.
- 103 Bialek P, Kern B, Yang X, Schrock M, Sosic D, Hong N, Wu H, Yu K, Ornitz DM, Olson EN, Justice MJ, Karsenty G: A twist code determines the onset of osteoblast differentiation. *Dev Cell* 2004;6:423–435.
- 104 Brueton LA, van Herwerden L, Chotai KA, Winter RM: The mapping of a gene for craniosynostosis: evidence for linkage of the Saethre-Chotzen syndrome to distal chromosome 7p. *J Med Genet* 1992;29:681–685.
- 105 Reid CS, McMorro LE, McDonald-McGinn DM, Grace KJ, Ramos FJ, Zackai EH, Cohen MM Jr, Jabs EW: Saethre-Chotzen syndrome with familial translocation at chromosome 7p22. *Am J Med Genet* 1993;47:637–639.

- 106 Reardon W, McManus SP, Summers D, Winter RM: Cytogenetic evidence that the Saethre-
Chotzen gene maps to 7p21.2. *Am J Med Genet* 1993;47:633–636.
- 107 van Herwerden L, Rose CS, Reardon W, Brueton LA, Weissenbach J, Malcolm S, Winter RM:
Evidence for locus heterogeneity in acrocephalosyndactyly: a refined localization for the Saethre-
Chotzen syndrome locus on distal chromosome 7p – and exclusion of Jackson-Weiss syndrome
from craniosynostosis loci on 7p and 5q. *Am J Hum Genet* 1994;54:669–674.
- 108 Chen ZF, Behringer RR: Twist is required in head mesenchyme for cranial neural tube morpho-
genesis. *Genes Dev* 1995;9:686–699.
- 109 Paznekas WA, Cunningham ML, Howard TD, Korf BR, Lipson MH, Grix AW, Feingold M,
Goldberg R, Borochowitz Z, Aleck K, et al: Genetic heterogeneity of Saethre-Chotzen syndrome,
due to TWIST and FGFR mutations. *Am J Hum Genet* 1998;62:1370–1380.
- 110 Kress W, Schropp C, Lieb G, Petersen B, Busse-Ratzka M, Kunz J, Reinhart E, Schafer WD, Sold J,
Hoppe F, Pahnke J, Trusen A, Sorensen N, Krauss J, Collmann H: Saethre-Chotzen syndrome
caused by TWIST 1 gene mutations: functional differentiation from Muenke coronal synostosis
syndrome. *Eur J Hum Genet* 2006;14:39–48.
- 111 Funato N, Twigg SR, Higashihori N, Ohyama K, Wall SA, Wilkie AO, Nakamura M: Functional
analysis of natural mutations in two TWIST protein motifs. *Hum Mutat* 2005;25:550–556.
- 112 de Heer IM, de Klein A, van den Ouweland AM, Vermeij-Keers C, Wouters CH, Vaandrager JM,
Hovius SE, Hooeboom JM: Clinical and genetic analysis of patients with Saethre-Chotzen syn-
drome. *Plast Reconstr Surg* 2005;115:1894–1905.
- 113 De Heer IM, Hooeboom AJ, Eussen HJ, Vaandrager JM, De Klein A: Deletion of the TWIST
gene in a large five-generation family. *Clin Genet* 2004;65:396–399.
- 114 den Dunnen JT, Antonarakis SE: Nomenclature for the description of human sequence variations.
Hum Genet 2001;109:121–124.
- 115 Jabs EW: TWIST and the Saethre-Chotzen syndrome; in Epstein C, Erickson R, Wynshaw-Boris A
(eds): *Inborn Errors of Development*, ed 1. Oxford, Oxford University Press, 2004, pp 401–409.
- 116 El Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Bourgeois P, Bolcato-
Bellemine AL, Munnich A, Bonaventure J: Mutations of the TWIST gene in the Saethre-Chotzen
syndrome. *Nat Genet* 1997;15:42–46.
- 117 Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, Ortiz de Luna RI, Garcia Delgado C,
Gonzalez-Ramos M, Kline AD, Jabs EW: Mutations in TWIST, a basic helix-loop-helix transcrip-
tion factor, in Saethre-Chotzen syndrome. *Nat Genet* 1997;15:36–41.
- 118 Bourgeois P, Bolcato-Bellemine AL, Danse JM, Bloch-Zupan A, Yoshida K, Stoetzel C, Perrin-
Schmitt F: The variable expressivity and incomplete penetrance of the twist-null heterozygous
mouse phenotype resemble those of human Saethre-Chotzen syndrome. *Hum Mol Genet*
1998;7:945–957.
- 119 El Ghouzzi V, Legeai-Mallet L, Aresta S, Benoist C, Munnich A, de Gunzburg J, Bonaventure J:
Saethre-Chotzen mutations cause TWIST protein degradation or impaired nuclear location. *Hum
Mol Genet* 2000;9:813–819.
- 120 Krebs I, Weis I, Hudler M, Rommens JM, Roth H, Scherer SW, Tsui LC, Fuchtbauer EM,
Grzeschik KH, Tsuji K, Kunz J: Translocation breakpoint maps 5 kb 3' from TWIST in a patient
affected with Saethre-Chotzen syndrome. *Hum Mol Genet* 1997;6:1079–1086.
- 121 Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, Ortiz de Luna RI, Garcia Delgado C,
Gonzalez-Ramos M, Kline AD, Jabs EW: Mutations in TWIST, a basic helix-loop-helix transcrip-
tion factor, in Saethre-Chotzen syndrome. *Nat Genet* 1997;15:36–41.
- 122 Holder N, Klein R: Eph receptors and ephrins: effectors of morphogenesis. *Development*
1999;126:2033–2044.
- 123 Wilkinson DG: Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev
Neurosci* 2001;2:155–164.
- 124 Murai KK, Pasquale EB: 'Eph'ective signaling: forward, reverse and crosstalk. *J Cell Sci*
2003;116:2823–2832.
- 125 Twigg SR, Kan R, Babbs C, Bochukova EG, Robertson SP, Wall SA, Morriss-Kay GM, Wilkie AO:
Mutations of ephrin-B1 (EFNB1), a marker of tissue boundary formation, cause craniofrontonasal
syndrome. *Proc Natl Acad Sci USA* 2004;101:8652–8657.

- 126 Wieland I, Jakubiczka S, Muschke P, Cohen M, Thiele H, Gerlach KL, Adams RH, Wieacker P: Mutations of the ephrin-B1 gene cause craniofrontonasal syndrome. *Am J Hum Genet* 2004;74:1209–1215.
- 127 Vasudevan PC, Twigg SR, Mulliken JB, Cook JA, Quarrell OW, Wilkie AO: Expanding the phenotype of craniofrontonasal syndrome: two unrelated boys with EFNB1 mutations and congenital diaphragmatic hernia. *Eur J Hum Genet* 2006;14:884–887.
- 128 Twigg SR, Matsumoto K, Kidd AM, Goriely A, Taylor IB, Fisher RB, Hoogeboom AJ, Mathijssen IM, Lourenco MT, Morton JE, et al: The origin of EFNB1 mutations in craniofrontonasal syndrome: frequent somatic mosaicism and explanation of the paucity of carrier males. *Am J Hum Genet* 2006;78:999–1010.
- 129 Wieland I, Reardon W, Jakubiczka S, Franco B, Kress W, Vincent-Delorme C, Thierry P, Edwards M, Konig R, Rusu C, et al: Twenty-six novel EFNB1 mutations in familial and sporadic craniofrontonasal syndrome (CFNS). *Hum Mutat* 2005;26:113–118.
- 130 Shotelersuk V, Siriwan P, Ausavarat S: A novel mutation in EFNB1, probably with a dominant negative effect, underlying craniofrontonasal syndrome. *Cleft Palate Craniofac J* 2006;43:152–154.
- 131 Compagni A, Logan M, Klein R, Adams RH: Control of skeletal patterning by ephrinB1-EphB interactions. *Dev Cell* 2003;5:217–230.

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