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FLRT2 Promotes Cellular Proliferation and Inhibits Cell Adhesion During Chondrogenesis

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ABSTRACT

One of the earliest events during chondrogenesis is the formation of condensations, a necessary pre-requisite for subsequent differentiation of a chondrogenic phenotype. Members of the Fibronectin Lecucine Rich Transmembrane (FLRT) proteins have been shown to be involved in cell sorting and neurite outgrowth. Additionally, FLRT2 is highly expressed at putative sites of chondrogenic differentiation during craniofacial development. In this study, we demonstrate that FLRT2 plays a role in mediating cell proliferation and cell–cell interactions during early chondrogenesis. Clones of stable transfectants of a murine chondroprogenitor cell line, ATDC5, were established in which FLRT2 was knocked down or overexpressed. Cells in which FLRT2 was knocked down proliferated at a slower rate compared to control wild-type ATDC5 cells or those containing a non-coding shRNA. In addition, FLRT2 knockdown cells formed numerous lectin peanut agglutinin (PNA) stained aggregates and exhibited higher expression of the cell adhesion molecule, N-cadherin. In an in vitro wound healing assay, fewer FLRT2 knockdown cells appeared to migrate into the defect. Surprisingly, the FLRT2 knockdown cells demonstrated increased formation of Alcian blue-stainable extracellular matrix, suggesting that their reduced aggregate formation did not inhibit subsequent chondrogenic differentiation. The opposite trends were observed in ATDC5 clones that overexpressed FLRT2. Specifically, FLRT overexpressing cells proliferated faster, formed fewer PNA-positive aggregates, accumulated increased Alcian blue-positive matrix, and migrated faster to close a wound. Collectively, our findings provide evidence for a role of FLRT2 in enhancing cell proliferation and reducing intercellular adhesion during the early stages of chondrogenesis. J. Cell. Biochem. 112: 3440–3448, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: ATDC5 CELLS; CELL–CELL INTERACTIONS; shRNA KNOCKDOWN; N-CADHERIN; CASPASE-3; CONDENSATIONS

Chondrogenesis comprises three major steps of commitment, mesenchymal cell condensation, and differentiation into cartilage [Goldring et al., 2006]. During the condensation phase, mesenchymal cells are dependent on increased mitotic activity and the migration of cells towards a center [Hall and Miyake, 2000]. These cells then aggregate to form chondrogenic and non-chondrogenic cell populations through the differential regulation and synthesis of specific adhesion molecules that change adhesive properties and mediate cell sorting [Tavella et al., 1994]. Members of the cadherin family of genes, especially N-cadherin, have been shown to play critical roles in cell–cell adhesion and signaling during formation of cellular condensations [Hall and Miyake, 2000]. Indeed, the correct spatiotemporal regulation of N-cadherin is critical for the initiation of chondrogenesis and temporally inappropriate cell adhesion activities are inhibitory to chondrogenesis [reviewed in Tuan, 2003]. The resulting cell–cell interactions trigger a cascade of cytoplasmic and nuclear events that activate the genes for cartilage-specific structural components. This culminates in secretion of an extracellular cartilage matrix composed chiefly of type II collagen and aggrecan.

Recent evidence points towards a possible role for FLRT2, a member of the Fibronectin (F) Leucine (L) Rich (R) Transmembrane (T) family, in mediating cell–cell interactions [Lacy et al., 1999; Bottcher et al., 2004; Karaulanov et al., 2006]. All three *FLRT* gene family members contain 10 leucine-rich repeats, a fibronectin-like III, and transmembrane domains with a short intracellular tail. FLRT3 has been shown to guide cell movements during gastrulation [Ogata et al., 2007], cell adhesion [Ogata et al., 2007; Egea et al.,

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2008; Maretto et al., 2008; Karaulanov et al., 2009], and neurite outgrowth [Robinson et al., 2004; Tsuji et al., 2004]. During craniofacial development, we showed that FLRT2 is expressed prior to, and during migration, of cranial neural crest cells to the developing mid-facial region [Gong et al., 2009]. It is these cranial neural crest-derived mesenchymal tissues that eventually condense at specific sites in the developing craniofacial region to form the cartilages and skeleton of the craniofacial region. For example, cranial neural crest cells condense within the single median frontonasal prominence to form the pre-cartilaginous nasal capsule that undergoes chondrogenesis to form the nasal septum [Scott, 1953]. Interestingly, *Flrt2* eventually becomes highly expressed in the chondrogenic nasal septal region [Gong et al., 2009], an area that has been proposed to play the role of pacemaker or growth center for the subsequent growth of the face and the skull and contribute to the overall changes in morphology observed during the development of the facial skeleton [Pavlov et al., 2003]. The fundamental mechanisms involved in the initiation, growth, boundary setting, and differentiation of the cartilaginous and skeletal structures of the frontonasal region are not well characterized. The distinct spatial and temporal expression of FLRT2 during craniofacial development led us to hypothesize that Flrt2 plays a role in mediating some critical events in chondrogenesis [Gong et al., 2009]. In the present study, we used a chondrogenitor cell line, ATDC5, to show that FLRT2 is involved in mediating critical events during early chondrogenesis such as proliferation and cell–cell interactions.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

ATDC5 cells were plated at a density of 2×10^5 in 60 mm tissue culture dish and cultured according to Shimizu et al. [2001]. Briefly, maintenance medium consisted of 1:1 ratio of DMEM/F12 supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml amphotericin B (Invitrogen). For induction of chondrogenesis, insulin–transferrin–selenium-X (Invitrogen) was added to a final concentration of 10 μg/ml insulin. Medium was changed every 2 days. Cells were harvested at pre-confluent (approximately 50% confluence), day 0 (85% confluence), and 4, 6, 7, 10, and 14 days, thereafter.

LENTIVIRUS-MEDIATED SMALL HAIRPIN RNA KNOCKDOWN OF FLRT2 IN ATDC5 CELLS

Five different commercially pre-synthesized small hairpin RNAs (shRNAs) against mouse FLRT2 were purchased (Sigma–Aldrich; product number: SHVRS) and tested. The regions chosen for the various shRNAs sequences were those having least homology with other members of the FLRT gene family. The constructed transfer vector, a vesicular stomatitis virus-G expression vector, and a gag-pol expression vector were co-transfected into 293T cells at a 1:1:1 molar ratio using Lipofectamine Plus (Invitrogen, Carlsbad, CA). The culture supernatant containing viral particles was harvested at 48 h after transfection, clarified with a 0.45 μm membrane filter (Nalgene, New York, NY), and stored at −70°C. Conditioned media containing the viral particles were used directly to infect ATDC5 cells in the presence of 8 μg/ml of polybrene (Sigma–Aldrich) for 8 h. As controls, a lentivirus preparation carrying a non-target "non sense" shRNA (pLKO.1-NT; Sigma–Aldrich) was used. Medium was changed, cells were grown until near confluence and passaged into 10 cm dishes, and puromycin (5 μg/ml) was added for selection. After wild-type ATDC5 cells had been killed, puromycin resistance colonies were collected separately and processed for immunoblotting analyses. Protein levels were quantitated by densitometric analysis. Only 2 of the 5 pre-synthesized FLRT shRNAs gave clones in which FLRT2 was appreciably knocked-down.

To establish ATDC5 cultures overexpressing FLRT2, we electroporated FLRT2 inserted in pcDNA™3.1D/V5-His-TOPO® vector. Stable transfectants of the overexpressing FLRT2 were selected with 5 μg/ml of G418.

WESTERN BLOT ANALYSIS

Cells were harvested at different time points of culture, specified in each experiment, and washed with PBS. Proteins were extracted by lysing cells with diluted 10× RIPA [200 mM Tris–HCl (pH 7.5); 1.5 M NaCl, 10 mM Na2EDTA; 10 mM EGTA; 10% NP-40; 10% sodium deoxycholate; 25 mM sodium pyrophosphate; 10 mM β-glycerophosphate; 10 mM Na_3VO_4; 10 μg/ml leupeptin] (Cell Signaling Technology), with a protease inhibitor, phenylmethylsulfonyl fluoride added just prior to use. Proteins were quantitated using the Bradford dye-binding method [Bradford, 1976; Bio-Rad protein assay). Twenty-five micrograms of total protein was loaded per well on 10% polyacrylamide gels, transferred onto a nitrocellulose membrane (GE Healthcare) and blocked for 1 h in 5% skim milk (BioShop) in Tris-buffered saline containing 0.01% Tween 20.

The following antibodies were used in this study: monoclonal anti-human Flrt2 (R&D Systems) 0.2 μg/ml; N-cadherin (1:200; MNCD2) [Matsunami and Takeichi, 1995] and GAPDH (1:10,000; Cell Signaling Technology). Primary antibody incubation was performed overnight at 4°C, followed by incubation with a 1:2,000 dilution of the secondary antibody for 1 h at room temperature. Signal was detected using ECL™ Western blotting detection reagents (GE Healthcare) according to the manufacturer's protocol and visualized on ChemiImager™ 5500 (R&D Systems). The data were analyzed by Quantity One software.

CELL COUNT

Cells were seeded at a density of 2×10^5 in 60 mm dishes. They were cultured and harvested at days 0 (85% confluent), 4, 7, and 14, and trypsinized. Trypan blue (0.4%) was added to specific dilutions of the cell suspension, loaded onto a standard hemacytometer, and counted to determine cell viability. All measurements were performed in triplicates.

IMMUNOHISTOCHEMISTRY

To monitor apoptosis, FLRT2 knockdown ATDC5 cells were collected after 14 days of incubation and immunostained for cleaved caspase-2 according to the method of Mai et al. [2010] using rabbit monoclonal antibody (Asp175) (5A1; 1:200, Cell Signaling).

LECTIN PEANUT AGGLUTININ (PNA) ANALYSIS

Cells of both FLRT2 knockdown and overexpressing groups with their controls were grown to about 80% confluence, trypsinized, and diluted to 2×10^7 cells/ml. Ten microliter drops of this cell suspension were plated onto culture dishes to establish high density micromass cultures. After 4 days of culture, cells were fixed in 4% paraformaldehyde at 4°C for 30 min. Cultures were then rinsed with PBS and incubated for 2 h in 50 μg/ml PNA (Sigma–Aldrich) diluted in PBS. Cultures were washed again with PBS, and the PNA was detected colorimetrically by DAB AEC (Vector Laboratories).

WOUND HEALING ASSAY

Cells were seeded at 1×10^6/60 mm plates and grown overnight to confluence. The monolayer cells were scratched with a 200-μl pipette tip to create a wound, washed twice with serum-free MEM to remove floating cells, and fresh medium added. Cells migrating from the leading edge were photographed and counted at 0 and 24 h after wounding.

ALCIAN BLUE STAINING

Cells grown for 14 days after confluence were rinsed with PBS, stained with 0.5% Alcian blue (pH 2.5) for half an hour, washed, and photographed. For Alcian blue quantification in the cultures, Alcian Blue stain was solubilized in 4 M guanidine HCl, 50 mM Tris–HCl (pH 7.4), 0.1% CHAPS. The absorbance of the solute was measured at a wavelength of 595 nm. Experiments were performed in triplicates.

REAL-TIME QUANTITATIVE (q)RT-PCR

Real-time RT-qPCR was conducted as outlined in Mai et al. [2010]. Primer sequences were: *Flrt2*—F: 5′-accagactggcagttctcaacgat-3′; R: 5′-tgtaatctgtcagccttgtctcct-3’; *Gapdh*—F: 5′-tgtccgtcgtggatctgac-3′; R: 5′-cctgcttcaccaccttcttg-3′.

RESULTS

FLRT2 IS EXPRESSED DURING EARLY CHONDROGENESIS IN ATDC5 CELLS

ATDC5 is a chondroprogenitor cell line derived originally from murine embryonic carcinoma [Atsumi et al., 1990]. These cells differentiate and undergo chondrogenesis in the presence of insulin [Shukunami et al., 1996]. ATDC5 expressed FLRT2 endogenously, even in maintenance medium prior to the addition of insulin (lane P, Fig. 1A). Upon the induction of chondrogenesis by the addition of insulin to the medium, these cells continued to express FLRT2 protein at a relatively high level until after days 4–7 (Fig. 1A,B, left graph), a period that corresponds to the onset of cell–cell interactions and the formation of cell condensations. At the mRNA level, *Flrt2* was expressed prior to the induction of chondrogenesis (Fig. 1B, right graph). In contrast to the protein level, however, the expression of *Flrt2* started to decrease almost immediately after insulin addition (day 0), reaching low levels by day 7 (Fig. 1B, right graph).

FLRT2 AFFECTS THE PROLIFERATION OF CELLS

The expression of FLRT2 prior to and during the onset chondrogenesis suggests a possible role in mediating early chondrogenic events. In order to study the function of this gene during chondrogenesis, we established stable transfectants of ATDC5 cells where FLRT2 was knocked down or overexpressed. Two clones overexpressed FLRT2 by 50% and 65% (clones Ov1 and Ov2, respectively; Fig. 2A) relative to control cells transfected with

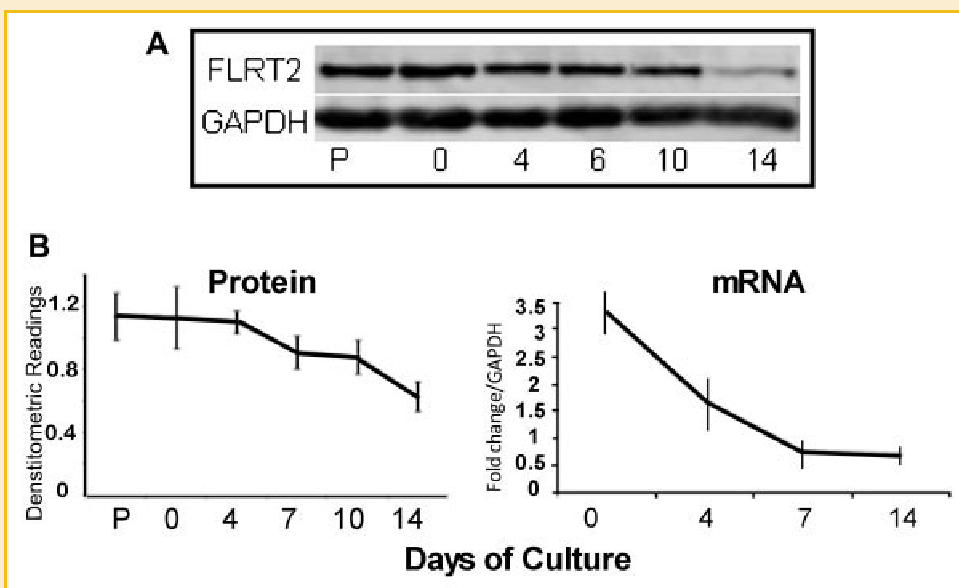

Fig. 1. Temporal expression of FLRT2 during chondrogenic differentiation of ATDC5 cells. A: FLRT2 immunoblots of proteins of cells harvested at pre-confluence (P; without addition of insulin to medium), 0–14 days of growth after addition of insulin. B: Left-Representative graph (out of three Western blots) of densitometric readings of FLRT2 protein levels relative to GAPDH from P, 0–14 days of culture. Right-Real-time RT-qPCR analysis of temporal expression of *Flrt2* mRNA (relative to GAPDH) from days 0 to 14 of culture.

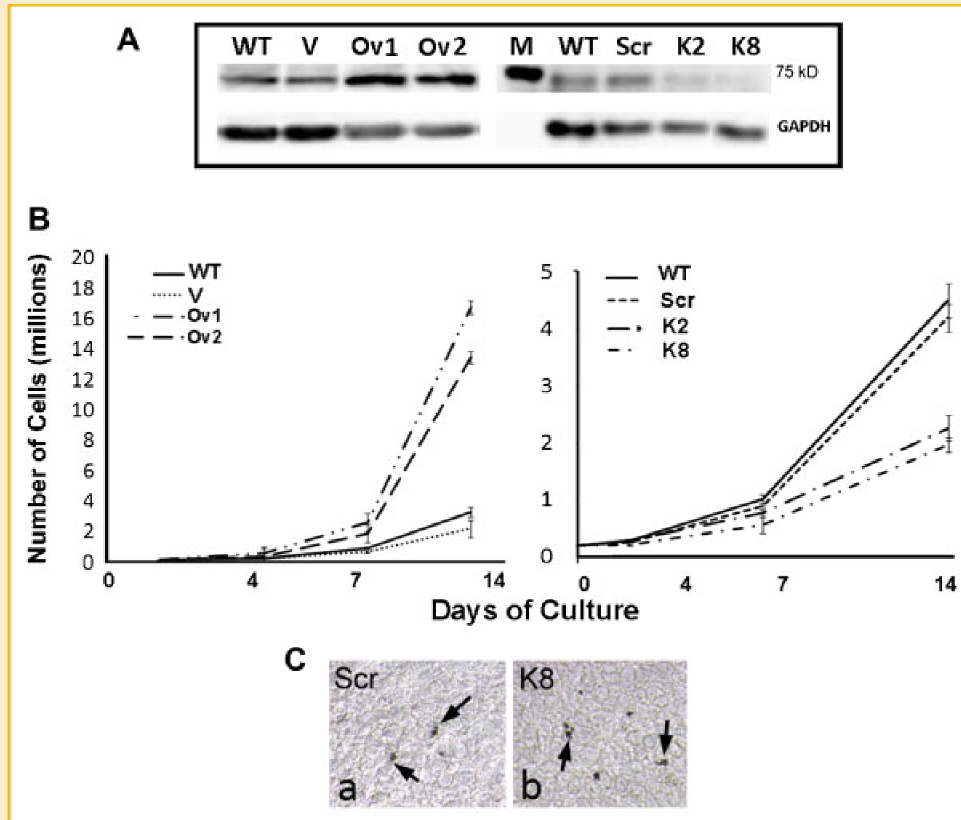

Fig. 2. Cell growth in FLRT2 stable transfectants. A: Western blot analysis with FLRT2 antibody on cell lysates of stable transfectants of FLRT2 overexpressing and knocked-down clones. B: Graphs illustrating the number of stable transfectants of FLRT2 overexpressing (left) and knocked-down (right) cells at 0–14 days of culture. C: Caspase-3 immunostaining of Scr and K8 knocked-down cultures grown for 14 days. Caspase-3 positive immunoreactive cells are visible as dark spots (demarcated by black arrows). W, wild-type; M, protein maker.

the empty vector (clone V, Fig. 2A). To knock down FLRT2 expression, we also established two ATDC5 clones that were stably transfected with Flrt2 shRNA expression constructs. Clones K2 and K8 exhibited 55% and 85% knockdowns in FLRT2 protein levels, respectively (Fig. 2A), relative to control cells infected with a non-coding shRNA (Scr).

During the course of establishing these stable transfectants, we observed a difference in their growth rates relative to control cells. We plated an equal number of cells from each group (day 0) and counted the cell number at days 4, 7, and 14 of culture in differentiation medium. In cultures that overexpressed FLRT2, the number of cells in clones Ov1 and Ov2 was significantly greater ($P < 0.005$) compared to either wild-type cells or cells containing the empty vector. The difference in number was especially striking by day 14 of culture (Fig. 2B, left graph). The positive effect of FLRT2 expression on cell numbers was corroborated by the observation of a significant decrease ($P < 0.005$) in the number of cells at days 4 onwards in ATDC5 clones in which FLRT2 had been knocked-down (Fig. 2B, right graph). Given the significant reduction in cell count in FLRT2 knockdown cultures, we considered whether increased cell death might contribute to the decreased cell numbers. We used activated caspase-3 immunostaining [Armstrong et al., 2011] to compare levels of apoptotic cell death in our FLRT2 knockdown clone K8 and the Scr control clones after 14 days of culture (Fig. 2Ca,b). Few caspase3-immunoreactive cells (arrows in Fig. 2Ca,b) were present in both control and K8 cultures, with no visible difference in the number of the immunoreactive cells between control and FLRT2 knockdown cultures. This indicates that apoptosis was not a major cause of the reduction in cell number in the FLRT2 knockdown cultures, and suggests that FLRT2 expression promotes increased proliferation of ATDC5 cells.

FLRT2 IS INVOLVED IN CELL–CELL AND CELL–MATRIX INTERACTIONS DURING CHONDROGENESIS

One early critical event during chondrogenesis is the formation of mesenchymal cell condensations necessary for the subsequent overt differentiation of chondroblasts. Members of the FLRT family of genes have been implicated in cell–cell interactions and cell sorting [Karaulanov et al., 2006; Ogata et al., 2007; Chen et al., 2009; Karaulanov et al., 2009]. Because FLRT2 was highly expressed during the early growth of the ATDC5 cells (Fig. 1) when extensive cell–cell interactions are occurring, we assayed the ability of ATDC5 cells to form condensations in micromass cultures of cells where FLRT2 was either knocked down or overexpressed. Pre-chondrogenic condensations express cell surface molecules that bind PNA lectin, allowing the condensations to be visualized [Hall and Miyake, 2000; Woods et al., 2007]. PNA staining of the FLRT2 knockdown micromass cultures grown for 4 days revealed the presence of highly

visible PNA-positive aggregates (white arrows in Fig. 3Ae), unlike FLRT2 overexpressing cultures where there was a relative absence of such aggregates (Fig. 3Ac). Within the dish, FLRT2 knockdown cells were distributed unevenly, with some areas containing thickened layers of cells and others devoid of cells (e.g., one such void area indicated by a black asterisk in Fig. 3Ae).

Cell adhesion molecules such as N-cadherin have been shown to be necessary for the formation of cell–cell contacts or condensations during chondrogenesis [Hall and Miyake, 2000; Tuan, 2003]. We hypothesized that the increase or decrease of aggregates observed in cells where FLRT2 was knocked-down or overexpressed, respectively, was due to changes in levels of cell–cell interactions mediated by N-cadherin. In FLRT2 overexpressing cultures where thick cell aggregations were not observed, the expression of N-cadherin protein was lower compared to wild-type cells or cells transfected with empty vector alone, especially at day 0 (Fig. 3B: left panel—representative N-cadherin immunoblot; right panel—quantitation of N-cadherin levels from two different experiments). By day 7, N-cadherin expression had declined to similar levels in all three groups (Flrt2 overexpressing, wild-type cells, and empty vector controls). In FLRT2 knockdown cultures where aggregates were highly visible, N-cadherin transcripts were expressed at a greater level compared to either wild-type cells or cells containing control shRNA (Fig. 3C).

To determine whether FLRT2 is involved in cell–matrix interactions, we conducted a cell-wounding assay using FLRT2 knockdown cells and FLRT2 overexpressors. At 24 h after scraping the culture with a pipette tip, fewer of the FLRT2 knockdown were observed to have migrated into the wound site (Fig. 4—outline of wound demarcated by vertical black lines. Compare K8 wound closure, f, with those of WT and Scr controls, j and k, bottom panel). In contrast, there was a significantly greater number of cells that migrated to close the wound in cultures of cells that overexpressed FLRT2 ($P < 0.005$; Fig. 4, top panel; compare f vs. d and e).

ALTERED LEVELS OF FLRT2 EXPRESSION AFFECT PRODUCTION OF SULFATED CARTILAGE MATRIX PROTEOGLYCANS

The preceding results indicated that FLRT2 mediates critical events at the onset of chondrogenesis, that is, cell proliferation and cell–cell adhesive interactions. To determine whether the altered levels of proliferation and cell–cell interactions had a subsequent effect on overt differentiation toward a chondrocytic phenotype, we used Alcian blue staining to determine whether FLRT2 knockdown and FLRT2 overexpression affected the amount of sulfated cartilage matrix proteoglycans accumulated after 14 days of culture. In cells that overexpressed FLRT2 (especially clone Ov2), Alcian blue staining appeared to be more intense relative to control cultures (Fig. 5A; compare d with a–c). Quantitative analysis showed a significant increase in the amount of Alcian blue staining in clone Ov2 (Fig. 5B, left graph). Clones K2 and K8, in which FLRT2 had been knocked-down by 55% and 85%, respectively, showed a "patchy" distribution of Alcian blue staining due to the presence of Alcian blue-positive nodules that were separated by areas of unstained cells (Fig. 5A; e–g). Quantitative analysis confirmed that there was a significant decrease in the amount of Alcian blue staining in the two groups of knockdown cells relative to cells expressing the scrambled shRNA control (Fig. 5B, right graph).

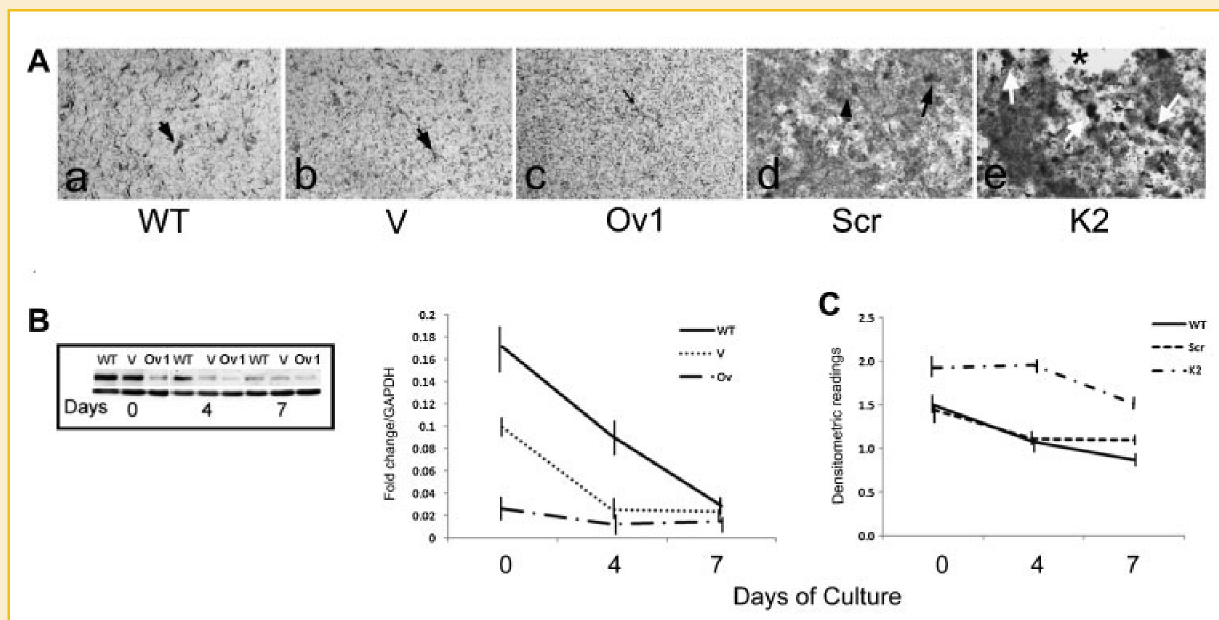

Fig. 3. PNA analysis and N-cadherin expression in FLRT2 stable transfectants. A: PNA staining of micromass cultures of FLRT2 overexpressing, Ov1 (c), and knocked-down, K2 (e), clones, with controls V (b), and Scr (d). Black (Aa–d) and white (Ae) arrows indicate areas of PNA-positive stained condensation. Black asterisk in Ae denotes an area on the dish devoid of cells. B. Western blot analysis of FLRT2 overexpressing cultures with antibody directed against N-cadherin at days 0, 4, and 7 of culture and quantitated (right graph, quantitation of one blot representative of three different experiments). C: Real-time RT-qPCR data of expression levels of *N-cadherin* FLRT2 knocked-down cultures.

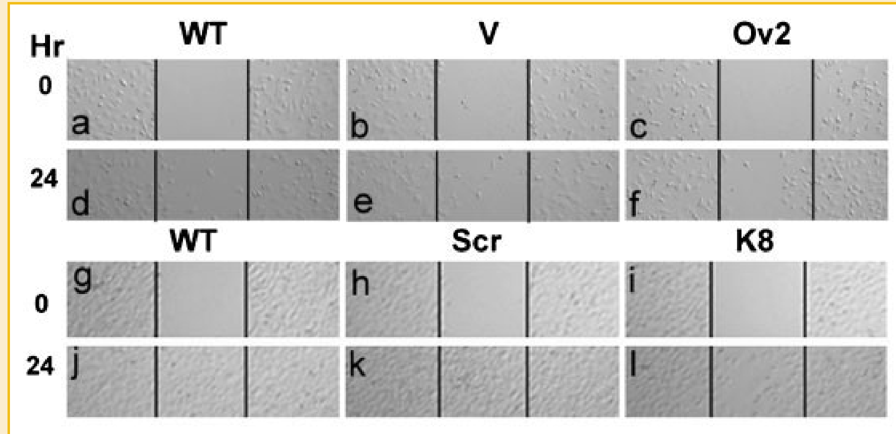

Fig. 4. Cell-wounding analysis in FLRT2 stable transfectants. FLRT2 overexpressing clone, Ov2 (top panel; c,f) and knocked-down clone, K8 (bottom panel; i,l) were grown to confluency and scratch marks were made with a pipette tip (0 h), the boundaries of which are demarcated by the black vertical lines in each image. Images were taken 24 h later with the scratch marks aligned as close as possible to the same position as when the first image was taken. Controls for the overexpressing group included WT (a,d) and V (b,e), and the knocked-down group WT (g, j) and Scr (h,k) at time 0 and 24 h after scratch marks were made.

DISCUSSION

Our analysis of the expression of FLRT2 in ATDC5 chondroprogenitor cells showed a difference in temporal expression at the protein and mRNA levels. The tapering off of *Flrt2 mRNA* levels soon after the induction of chondrogenesis was in contrast to the longer duration of FLRT2 protein expression (from pre-confluence up to days 4–7 of culture). Members of the FLRT family are transmembrane proteins [Bottcher et al., 2004; Robinson et al., 2004; Haines et al., 2006], suggesting the possibility that FLRT2 protein might be retained on the cell membrane and remains functionally active for a few days after depletion of its mRNA transcripts. Based on its temporal pattern of expression, we postulated that FLRT2 mediates events during the onset of chondrogenesis. Our functional analysis using FLRT2 overexpression and shRNA-mediated FLRT2 knockdown in ATDC5 chondroprogenitor cells has, indeed, revealed two critical roles for FLRT2 during early chondrogenesis.

Firstly, we showed that FLRT2 *increased* the rate of proliferation of ATDC5 chondroprogenitor cells. Cells overexpressing FLRT2 proliferated faster whereas cells containing knockdown of FLRT2 proliferated at a slower rate (summarized in Fig. 6). It is interesting to note the depressive effect of FLRT2 knockdown on cell proliferation continued even to day 14 of culture, when FLRT2 levels were already on the decline in control ATDC5 cells. Beyond day 14, however, we observed a catch-up in the rate of proliferation of the FLRT2 knockdown cells compared to controls (data not shown), suggesting that factors other than FLRT2 may play a role in mediating the rate of cell proliferation at later stages of chondrogenesis. In this regard, it is worth noting that FLRT proteins have been associated with *Fgf*

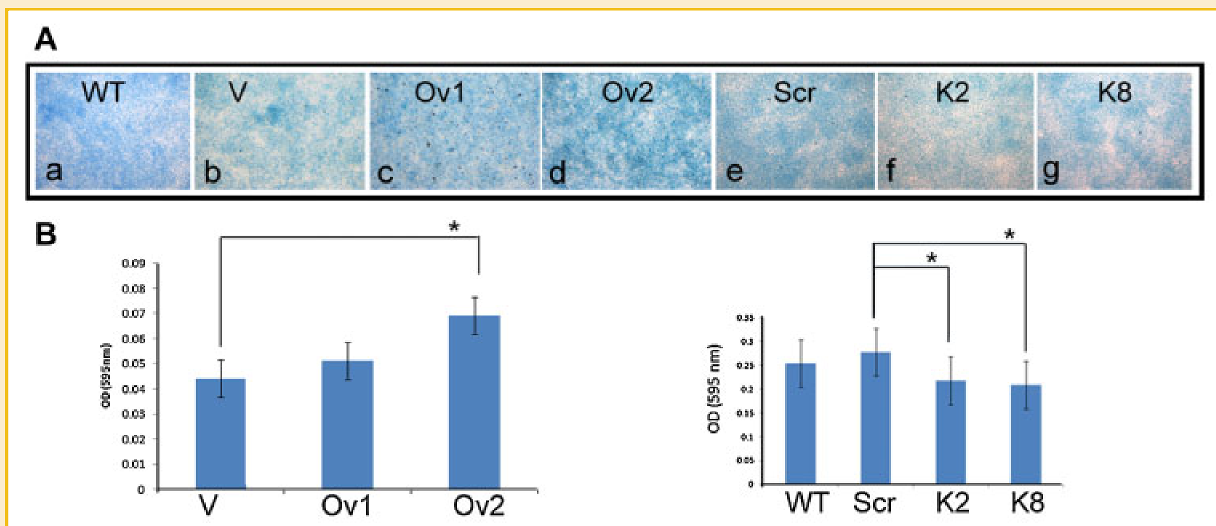

Fig. 5. Alcian blue staining of stable transfectants of FLRT2. A: Alcian blue analysis of cells in which FLRT2 had been knocked-down (K2, K8) or overexpressed (Ov1, Ov2) and grown for 14 days. B: Quantitative analysis of Alcian blue staining in both groups (done in triplicates).

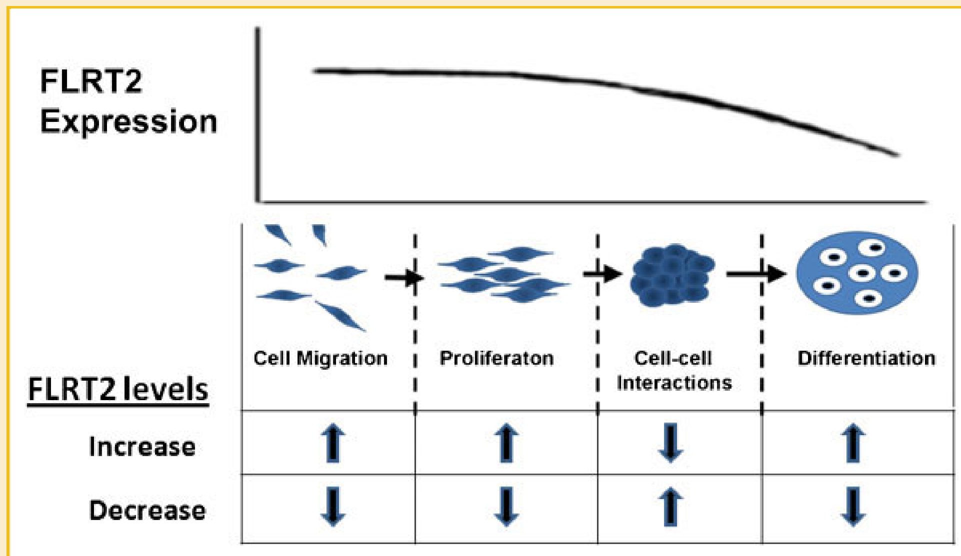

Fig. 6. Summary of FLRT2 function during chondrogenesis. Schematic diagram showing the temporal expression of FLRT2 (top) across the four phases of development of a chondrogenic differentiation program (as outlined in schematic diagram in middle), represented from left to right: cell migration, proliferation, cell–cell interactions, and differentiation to a chondrogenic phenotype. The bottom panel summarizes the results of FLRT2 overexpression ("Increase") and knockdown ("Decrease") on the four phases of chondrogenesis. An upward facing arrow ↑, indicates a positive, and a downward facing arrow ↓, a negative effect on the specific phase of chondrogenesis as outlined in the middle diagram. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

signaling [Bottcher et al., 2004; Haines et al., 2006], known to have a stimulatory effect on the proliferative activity on chondrocytes [Goldring et al., 2006]. Furthermore, we have also shown specifically that FLRT2 interacts with FGFR2 [Wei et al., 2011], making it likely that *Fgf* signaling may be connected to the activities regulated by FLRT2.

The second role of FLRT2 identified in this study is an *anti-adhesive* function during chondrogenesis. We showed that knocking down the level of FLRT2 protein increases N-cadherin-mediated cell aggregate formation, whereas overexpression of FLRT2 decreases these adhesive interactions (summarized in Fig. 6). Our observations are consistent with a previous report that the structurally related FLRT3 protein inhibits cadherin-mediated cell adhesion in *Xenopus* embryos [Ogata et al., 2007], a finding consistent with what we observed with FLRT2 during chondrogenesis. Additionally, *Xenopus FLRT3* has been shown to be involved in cell sorting [Karaulanov et al., 2006] and cell adhesion [Ogata et al., 2007; Karaulanov et al., 2009]. The anti-adhesive property of FLRT2 is likely mediated by the 10 Leucine Rich Repeat domains in the *extracellular* portion of the FLRT2 protein, as had been demonstrated for FLRT3 [Karaulanov et al., 2006]. The *intracellular* domain of FLRT3 directly interacts with Rnd1, a small GTPase, to decrease cell adhesion by controlling cell surface levels of cadherin through a dynamin-dependent endocytosis pathway [Ogata et al., 2007]. Rnd1 also binds to Unc5B, a netrin receptor (which also interacts directly with the ectodomain of FLRT3) to synergistically induce cell deadhesion in *Xenopus* embryos [Karaulanov et al., 2009]. Paraxial protocadherin (PAPC) counters the FLRT3 function in adhesion by inhibiting the recruitment of the Rnd1 to FLRT3 [Chen et al., 2009]. Therefore, FLRT3 modulates two opposing activities within the same protein: homotypic cell sorting/adhesion via the ectodomain [Karaulanov et al., 2006] *and* cell deadhesion via the intracellular tail [Ogata et al., 2007; Chen et al., 2009; Karaulanov et al., 2009] through interactions with other proteins. It is highly possible that a similar mechanism of action exists for FLRT2 during chondrogenesis. Interestingly, *Unc5C* is one of the genes that we have identified, together with *Flrt2*, in a microarray screen conducted to reveal novel molecular players in the developing mid-facial region [S.-G. Gong, unpublished data].

The two functions of FLRT2 (regulation of proliferation rate and cell adhesion) fit well with our initial hypothesis, based on its in vivo expression pattern in early murine embryos [Gong et al., 2009], of a possible functional role for FLRT2 in regulating the proliferation and motile behavior of cranial neural crest cells during the early stages of their formation and dorso-ventral migration in the developing embryo. Once cranial neural crest cells reach their final destinations in the developing branchial regions, condensation occurs at specific sites in the craniofacial region [Moore and Persaud, 1998]. Condensation is a pivotal stage in cartilaginous and skeletal development [Hall and Miyake, 1995, 2000] and the extent of cell condensation has been correlated with the level of chondrogenesis [San Antonio and Tuan, 1986]. Therefore, FLRT2 may have a significant role in determining the boundaries of the future chondrogenic areas of the mid-facial by regulating the extent of adhesion/deadhesion. By defining the size and sites of condensation in the developing mid-facial region, FLRT2 may be a significant player in regulating the shape and patterning of the developing mid-facial region, where it is highly expressed. Although a *Flrt2* knockout mouse does exist, the majority of *Flrt2* mutant embryos die between E11.5 and E12.5 as a result of placental or cardiovascular defects [Muller et al., 2011], prior to the onset of chondrogenic differentiation in the developing mid-facial region. A

conditional knockout targeting specifically cranial neural crest cells (e.g., under the *Wnt1* promoter) might be more appropriate to study the effects of *Flrt2* knockouts in the developing craniofacial region.

We found that FLRT2 knockdown cultures, in spite of having more cellular aggregates, expressed statistically smaller quantities of sulfated cartilage matrix proteoglycans (as measured by Alcian blue staining). The decreased proliferative rate of FLRT2 knockdown cells did not seem to affect the formation of aggregates, a finding supported by a previous study that showed that cell sorting mediated by FLRTs was independent of cell proliferation [Karaulanov et al., 2006]. In the normal state during limb bud development, for example, condensations during chondrogenesis form as a result of an increase in mesenchymal cell-packing density without an increase in cell proliferation [Janners and Searls, 1970; Thorogood and Hinchliffe, 1975]. It can be argued, therefore, that condensation alone, in the absence of certain factors, for example, SOX 9 or an adequate number of cells, is not enough to push the transition to a differentiation program. Therefore, although FLRT2 expression declines during the late stages of chondrogenesis, FLRT2 nonetheless appears to have a role in determining the final outcome of the chondrogenic differentiation program. Further work will be needed to characterize the contributions of each specific biological event, for example, proliferation, cell–cell interactions, matrix deposition, towards the outcome of the overall chondrogenic program. The specific abilities of FLRT2 in increasing the rate of proliferation *and* decreasing adhesive interactions between chondroprogenitor cells offer an opportunity to dissect how these two biological processes intertwine to generate the final differentiated chondrogenic state. Information derived from studies on FLRT2 will ultimately contribute towards improved understanding of the cellular and molecular bases of human skeletal and cartilaginous diseases.

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