The Effect of Activating Fibroblast Growth Factor Receptor 3 Mutations on Osteogenic Differentiation and Ectopic Bone Formation by Human Periosteal Derived Cells

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Abstract

Activating mutations in Fibroblast Growth Factor Receptor 3 (FGFR3) have previously been shown to cause skeletal dysplasias through their effect on growth plate chondrocytes. However, the effect of FGFR3 mutations on bone progenitor cells may differ. The objective of this study was to investigate the effect of specific activating FGFR3 mutations on osteogenic differentiation in vitro and in vivo bone formation by periosteal derived cells (PDCs) seeded on calcium phosphate/collagen scaffolds.

PDCs were isolated from hypochondroplasic (N540K mutation) and achondroplasic (G380R mutation) patients, along with age/sex matched controls. These cells were characterised in vitro for proliferation, osteogenic differentiation, FGFR3 signalling and in vivo bone formation. Subsequently, empirical modelling was used to find correlations between in vivo formed bone and in vitro cell behaviour. These data showed that in contrast to the G380R mutation, which produced no bone, the N540K mutation induced significant ectopic bone formation on specific carriers. This allowed correlation between percentage of induced bone formation to elevated proliferation and differentiation. Correlating osteogenic markers included Collagen type 1, alkaline phosphatase and osteocalcin. Enhanced proliferation was attributed to increased phosphorylation of Erk-1/2.

This study highlights the importance of FGFR3 in periosteal cell differentiation and also indicates it potential for targeted tissue engineering strategies.

Keywords: FGFR3; Periosteal derived cells; Osteogenic differentiation; Bone formation

Introduction

The importance of the Fibroblast Growth Factor (FGF) receptor 3 (FGFR3) signalling pathway in endochondral bone development, and more specifically in the regulation of chondrocyte proliferation and differentiation in the epiphyseal growth plate, was discovered when an activating mutation in FGFR3 was found to cause achondroplasia, the common form of human dwarfism [1]. Even though the mechanism of action of these mutations is not fully understood, pathways involved in FGFR3 signalling such as the STAT and Mitogen Activating Protein Kinase (MAPK) pathways have been suggested to play a role [2-4]. Inhibition of chondrocyte proliferation is caused by induced phosphorylation of STAT-1, which after translocation into the nucleus induces transcription of cell cycle inhibitors such as p21 [5]. In addition, the MAPK-pathway has been suggested to play a role due to phosphorylation of Erk1/2 and p38 [6-8]. The latter may cause premature and enhanced osteoblast differentiation of cells carrying activating mutations in FGFR3. This was confirmed in a study by Su et al., where enhanced phosphorylation of Erk-1/2 was suggested to be responsible for elevated cell proliferation together with impaired bone matrix mineralisation [9]. Phosphorylation of p38 in the same study was correlated to promotion of osteogenic differentiation but reduction in proliferation and cell induced mineralisation.

The location of the mutation may be crucial for the severity of resulting skeletal dysplasia. The most common mutation, present in 98% of the cases of achondroplasia, is the G380R substitution in the transmembrane domain [10]. This causes increased stabilisation and accumulation at the cell surface thus promoting uncontrolled, prolonged, ligand-dependent activation of the receptor. Another common activating mutation in FGFR3 is the N540K mutation, detected in 60-65% of patients diagnosed with the less severe hypochondroplasia. This mutation is located in the intracellular Tyrosine Kinase (TK)-1 domain of the receptor [11], and has therefore been suggested to give an altered gene-expression pattern compared to mutations in other domains, such as the transmembrane domain. In addition, the N540K mutation displays a ligand-independent phosphorylation of the TK domain, leading to activation of the receptor in absence of a binding ligand.

To assess the influence of FGFR3 mediated signalling on bone progenitors, we herein analyse Periosteal Derived Cells (PDC) isolated from patients with achondroplasia and hypochondroplasia in vitro and in vivo. Together with high proliferative and osteochondrogenic differentiation profiles in these cells [12,13], the location of the periost suggests these cells to be early contributors in fracture repair and are therefore deemed to mediate the primary steps of ossification in the fracture haematoma [14,15]. We herein demonstrate that cells containing the N540K mutation display increased proliferation and
enhanced osteogenic differentiation, which could be correlated to in vivo bone formation.

This work represents a first step to elucidate the effect of FGFR3 signalling on PDC dynamics and bone formation.

Materials and Methods

Isolation of periosteal derived cells

Isolation of PDCs from four donors as follows; N540K: 5 year old male, G380R: 5 year old male, Control 1: 3 year old female, Control 2: 8 year old male, was carried out as described by De Bari et al. [12]. Cells were expanded in growth medium (GM) consisting of Dulbecco’s Modified medium (DMEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS (Gibco, Merelbeke, Belgium) and antibiotic-antimycotic solution (100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B; Invitrogen, Merelbeke, Belgium) until passage 5. The ethical committee for Human Medical Research (KU Leuven) approved all procedures and the patient informed consents were obtained.

Analysis of cell metabolism and FGFR3 signalling

Metabolic activity was measured by incubation of monolayer cultures with 10% Alamar Blue® (Invitrogen, Merelbeke, Belgium) in GM. Fluorescent signal was measured and values from each time point were normalised to day 1 for respective cell population. FGFR3 signalling was analysed within in vitro cultured proliferating cells by Western blot. Briefly, the cells were lysed in protein extraction buffer containing 0.3 M PMSF and Protease Inhibition Cocktail (Sigma, Bornem, Belgium). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo SCIENTIFIC, Erembodegem, Belgium) and 10 μg was loaded on NuPAGE 4-12% Bis-Tris gel (Invitrogen, Merelbeke, Belgium). Antibodies were diluted according to the manufacturer’s instructions: Phospho-STAT1 (Tyr701 (58D6)) rabbit monoclonal, Phospho-p38 rabbit monoclonal, Phospho-STAT1 (E10) mouse monoclonal (Cell Signalling Technology). GAPDH was used to assess equal loading of proteins, images were developed by a LAS3000 Imaging System (FUJI).

Osteogenic differentiation

To analyse the differentiation capabilities of isolated cells, monolayer cultures were stimulated for 21 days with osteogenic media (OM; GM containing 100 nM dexamethasone, 0.25 mM ascorbic acid and 10 mM β-Glycerophosphate). As control, cells were cultured in parallel in GM. RNA extraction was performed using the RNeasy Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. Complementary DNA (cDNA) was obtained by reverse transcription of 500 ng total RNA with Oligo (dT) primer (Superscript III; Invitrogen, Merelbeke, Belgium). Quantitative PCR was performed using a Rotor-Gene 6000 system (Corbett, Westburg, Leusden, and The Netherlands) with a SybrGreen detection system (Applied Biosystems, Halle, Belgium). The two step reaction, 94°C for 3 s and 60°C for 40 s, was cycled 40 times and relative gene expression calculated using the calculated 2-ΔΔCT method.

In vivo osteogenesis

To analyse the bone forming potential of cell populations, two different clinically used bone void fillers, composed of calcium phosphate (CaP) [16] particles in an open collagen network (CopiOs™ (Zimmer, Wemmel, Belgium) and Collagraft™ (Neucoll, Cambell, California, US)), were selected based on their different properties and bone forming capacity in combination with hPDCs [17]. Each material was punched into 21 mm³ cylindrical scaffolds. 1 million cells applied to the upper surface of each scaffold. Seeded scaffolds were incubated overnight at 37°C to allow cell attachment, and thereafter implanted subcutaneously in the back at the cervical region of NMRI-nu/nu mice. Implants were collected 56 days post implantation, each scaffold was fixed in 4% formaldehyde, decalcified in EDTA/PBS (pH 7.5) for 2 weeks, paraffin embedded and processed for histology. All procedures were approved by the local ethical committee for Animal Research (KU Leuven). Histological examination and bone quantification were carried out as previously described [17].

Multivariate analysis and partial least-squares regression modelling

In a multivariate analysis pairwise correlations between all variables from in vitro and in vivo measurements were investigated using the Pearson product-moment correlations for each pair of variables. Correlations were calculated by pairwise deletion method and significant probabilities were identified for p-values < 0.05.

In order to determine whether multiple molecular signals in combination could quantitatively predict the in vivo bone formation response of different cell types, a mathematical formalism, partial-least square regression (PLSR), previously shown to be capable of relating quantitative contributions of multiple signals to a (single) measured response [18,19], was used. PLSR is able to identify the information content within the set of measured molecular signals that most closely maps onto the output response (amount of bone formation). The resulting mapping of lumped signals to corresponding responses allows to identify the most "important variables" for the in vivo bone formation by hPDCs in Copios™ and Collagraft™ carriers. Each cell population was individually withheld from the training set to construct a cross-validation model to minimise the risk of over fitting. To perform the Multivariate Analysis, correlation clustering and to generate the PLSR model, the software package JMP® 8 (SAS Institute Inc., Cary, NC) was used.

Statistical analysis

Data expressed as mean ± SEM. Statistical significance was determined using student T-test to compare between independent groups. Statistical significance is indicated on all graphs as follows: *: p < 0.05; **: p < 0.01; ***: p < 0.001 (n=3).

Results

Activating FGFR3 mutation increases periosteal cell proliferation

Metabolic activity was measured as an indication of cell proliferation on cells containing an activating mutation in the transmembrane- and the TK1-domain of FGFR3; G380R and N540K respectively along with controls. As seen in Figure 1A, cells commonly keep a linear growth until day 11, the time point when cells reach confluence. N540K cells displayed highest metabolic activity until day 11, the time point when cells reach confluence. N540K mutant cells displayed a similar metabolic profile as its age-matched control, Control 2.

To investigate whether FGFR3 signalling was associated with alteration in cell proliferation observed in N540K mutant cells, phosphorylation status of FGFR3 signalling proteins was analysed (Figure 1B), where phosphorylation of STAT1 was similar in all four
cell populations. The MAPK-pathway member p38 is a known inducer of cell differentiation. Both FGFR3 mutants N540K and G380R displayed an elevated phosphorylation of p38 when compared to control cell populations. However, only N540K displayed high levels of phosphorylated Erk1/2, a MAPK associated with cell proliferation.

**Increased osteogenic differentiation with the N540K mutation**

The effect of FGFR3 mutations on osteogenic differentiation of PDCs was investigated by gene expression of bone markers such as Sox9, Runx2, alkaline phosphatase (ALP), collagen type 1 (Coll1) and osteocalcin. A significant induction in the expression of FGFR3 was observed in N540K cells during osteogenic differentiation together with the G380R cells (Figure 2A). No induction in FGFR3 expression was observed in control cell populations.

Osteogenic stimulated cells from all cell populations displayed a significant decrease in expression of a chondrogenic transcription factor, Sox9 (Figure 2B). Conversely, the osteogenic marker Runx2 was significantly up regulated in N540K, G380R and control 2 (Figure 2C). Expression of the early osteoblast marker ALP was significantly increased in all cell populations when stimulated with OM, and the elevation was a 5 fold higher in the N540K mutant (Figure 2D). Coll1, an early/mid marker of osteogenic differentiation, showed elevated expression levels in stimulated N540K mutant and Control 2 as compared to unstimulated cells. In contrast, expression of Coll1 in the G380R mutant and control 1 was down regulated in stimulated cells (Figure 2D). Expression of the late osteogenic marker, osteocalcin, increased 1.6 fold in stimulated N540K cells, G380R, control 1 and control 2 displayed minor changes in expression, in comparison to unstimulated cells.

**Activating FGFR3 mutations affect the ectopic bone forming capacity of periosteal cells**

To investigate the specific effect on ectopic in vivo bone forming capacity between the two mutants N540K and G380R, and control populations, cells were seeded on two different CaP-carriers with either known bone forming capacity (CollagraftTM) or poor bone forming capacity (CopiosTM) in combination with hPDCs. Histomorphometry revealed that a range in quantity of de novo formed bone was observed in combination of both carriers and cell populations (Figure 3). N540K, control 1 and control 2 could be induced to induce ectopic bone formation within the CopiosTM material. The amounts of bone were quantified to (Figure 3A–3D, 3I): Control 1: 0.03± 0.017%, Control 2: 2.11±0.51%, N540K: 31.5±2.5%, G380R: 0±0%. When cells were seeded on the CollagraftTM carrier, all cell population displayed the ability to form bone to various extents as (Figure 3E–3H, 3I): Control 1: 0.03±0.017%, Control 2: 3.89±0.50%, N540K: 15.13±1.75%, G380R: 0.57±0.19%. The N540K mutation results in a significant increase in bone formation of 15 fold in Copios (BV%)–pERK1/2: p=0.0374, p38–FGFR3: p=0.0222, p38–p21: p=0.0390, p38–Sox9: p=0.0276).

The variable importance plot (Figure 4B) generated by a first PLSR model including all in vitro measurements indicates that only six out of the 11 variables are important to obtain a good prediction. Variables with an importance larger than 0.8 have been retained for the second PLSR model. In the final model, a combination of five molecular signals (ALP, Coll1, osteocalcin, pERK1/2 and Metabolic Activity Day 6, all with a positive effect on the predicted amount of in vivo bone formation, together with p-value < 0.05 (Copios(BV%))–Collagraft (BV%): p=0.0141,
ECTOPIC BONE FORMATION IN COMBINATION WITH CaP-CARRIERS IN TM

HYPOCHONDROPLASIA DUE TO IMPAIRED CHONDROGENESIS [10, 11]. IN THIS

DISCUSSION

FORMATION) IS CAPABLE OF PREDICTING THE QUANTITY OF BONE FORMED IN VIVO WITH AN ACCURACY OF 93.56% FOR ONE PRINCIPAL COMPONENT (FIGURE 4C).

ACTIVATING MUTATIONS IN FGFR3 MAY CAUSE SHORT LIMB BUD FORMATION, WHICH CAN RESULT IN DISORDERS LIKE HYPOCHONDROPLASIA AND HYPOCHONDROPLASIA DUE TO IMPAIRED CHONDROGENESIS [10, 11]. IN THIS STUDY, WE INVESTIGATED THE EFFECT OF SPECIFIC MUTATIONS ON PERIOSTEAL CELL

DYNAMICS. THIS WAS CARRIED OUT BY ANALYSING METABOLIC ACTIVITY, AFFECTED SIGNALLING PATHWAYS AND IN VIVO ECTOPIC BONE FORMATION.

UPON INVESTIGATION OF METABOLIC ACTIVITY, PREVIOUSLY USED AS A MEASURE OF CELL PROLIFERATION [20], CELLS CARRYING THE N540K MUTATION DISPLAYED A HIGH PROFILE WITHIN THE CULTURES UNTIL DAY 11. THE INCREASED METABOLIC ACTIVITY IN THE N540K MUTANT, WHEN COMPARED TO ALL OTHER CELL POPULATIONS, APPEARS TO BE THE OPPOSITE OF WHAT HAS PREVIOUSLY BEEN SHOWN IN ACTIVATING FGFR3 MUTANTS IN THE GROWTH PLATE IN VIVO [6]. THIS RESULT MAY BE DUE TO THE FACT THAT WE ARE WORKING WITH A PROGENITOR CELL POPULATION AND NOT DIFFERENTIATING CHONDROCYTES, SEEN IN THE IN VIVO GROWTH PLATE WHERE ACTIVATING FGFR3 MUTATIONS MAINLY AFFECT PROLIFERATING AND DIFFERENTIATING CHONDROCYTES. ADDITIONALLY, RECENT WORK ON BONE NARROW STROMAL CELLS, WHICH MAY REFLECT PDC BIOLOGY BETTER THAN CHONDROCYTES, SUGGEST THAT ACTIVATING MUTATIONS IN THE TRANSMEMBRANE DOMAIN RESULT IN A DECREASED CELL PROLIFERATION, WHICH IS PARTIALLY REFLECTED IN OUR DATA IN G380R [9]. TO OUR KNOWLEDGE NO DATA IS AVAILABLE ON THE EFFECT OF THE N540K MUTATION, BUT DUE TO THE ALTERED MECHANISM OF LIGAND INDEPENDENT ACTIVATION, THIS MUTANT MAY INDEED FUNCTION DIFFERENTLY COMPARED TO TRANSMEMBRANE MUTATIONS. TO IDENTIFY WHETHER FGFR3 SIGNALLING WAS CAUSING THE ALTERED PROLIFERATIVE PROFILE, ACTIVATION OF DOWNSTREAM FGFR3 SIGNALLING MODULATORS WAS INVESTIGATED. PHOSPHORYLATION OF STAT1 HAS PREVIOUSLY BEEN SUGGESTED A ROLE IN HIBITION OF CHONDROCYTE PROLIFERATION [21], THE LACK OF DIFFERENCE IN PROTEIN ACTIVATION MAY EXPLAIN THE FAILURE TO OBSERVE A DECREASED PROLIFERATION WHEN COMPARING THE FGFR3 MUTANT CELL POPULATIONS WITH CONTROLS. N540K AND G380R MUTANTS INDUCE ACTIVATION OF p38, WHICH HAS PREVIOUSLY BEEN SHOWN TO ALLOW THE PROMOTION OF OSTEOGENIC DIFFERENTIATION [9]. ADDITIONALLY, N540K SHOWED ENHANCED PHOSPHORYLATION OF ERK1/2, WHICH HAS BEEN IMPLICATED IN THE INDUCTION OF CELLULAR PROLIFERATION [9], AND MAY INDICATE WHY THIS MUTATION IS ASSOCIATED WITH A HIGHER PROLIFERATIVE CAPACITY OF PDCS IN VITRO UNDER THE EXPERIMENTAL CONDITIONS USED.

TO INVESTIGATE THE OSTEOGENIC POTENTIAL OF THE STUDIED CELL TYPES, OSTEOGENIC MARKER EXPRESSION WAS ANALYSED IN POPULATIONS OF UNSTIMULATED AND OSTEOGENIC STIMULATED CELLS. AN ENHANCED EXPRESSION OF FGFR3 mRNA TRANSCRIPTS WAS OBSERVED IN STIMULATED CELLS IN BOTH FGFR3 MUTANT POPULATIONS, IN LINE WITH LITERATURE [22]. THIS CAN BE CORRELATED TO WORK BY MATSUSHITA ET AL. [8] THAT REPORTED AN ELEVATED EXPRESSION OF FGFR3 IN CHONDROCYTES WITH AN ACTIVATING MUTATION IN FGFR3. THIS LED TO PREMATURE SYNCHONDROSIS CLOSURE AND INCREASED OSTEOBLAST DIFFERENTIATION IN AREAS SURROUNDING THE CLOSURE. INTERESTINGLY, THIS WAS ASSOCIATED WITH INCREASED BONE FORMATION [8]. THIS IS ESPECIALLY REMARKABLE AS THE N540K MUTANT INDUCED SIGNIFICANT IN VIVO ECTOPIC BONE FORMATION IN COMBINATION WITH CaP-CARRIERS IN THIS STUDY.

SOX9 IS EXPRESSED IN PROLIFERATING AND DIFFERENTIATING CHONDROCYTES UNTIL THE FINAL STAGE OF HYPERTHESIS IS REACHED AND IS THEREFORE A MARKER WHICH PROVIDE AN INDICATION WHETHER CELLS ARE IN THE CHONDROGENIC LINEAGE [23]. THEREFORE, THE DECREASE IN SOX9 EXPRESSION OBSERVED IN OSTEOGENIC STIMULATED CELLS INDICATES THAT THE CELLS ARE NOT ENTERING THE CHONDROGENIC LINEAGE [24, 25]. THIS IS CONFIRMED BY RUNX2 EXPRESSION, AS RUNX2 IS KNOWN TO PROMOTE DIFFERENTIATION INTO THE MATURE OSTEOBLAST AND THEN FURTHER INTO THE OSTEOCYTE TERMINATION [26, 27]. THE EXPRESSION OF RUNX2 IS ENHANCED IN BOTH FGFR3 MUTANTS CULTURED IN CONDITIONS PROMOTING OSTEOGENESIS, COMPARED TO UNSTIMULATED CELLS. THE EXPRESSION IS HIGHEST IN N540K, AND LEADS TO THE HYPOTHESIS THAT THIS MUTATION ALLOWS AN INCREASED SUSCEPTIBILITY FOR OSTEOGENIC DIFFERENTIATION. THIS HYPOTHESIS IS STRENGTHENED BY THE INCREASED EXPRESSION OF ADDITIONAL OSTEOGENIC MARKERS SUCH AS ALP, COL11 AND

Figure 3: Analysis on ectopic bone formation in vivo. Hematoxylin & Eosin staining display newly formed bone were cell nuclei are detected with blue colour. A-O show newly formed bone induced by cells seeded on a Copios™ carrier. A: Control 1, B: Control 2, C: N540K, D: G380R. E-H: display newly formed bone induced by cells seeded on Collagraft™ carrier. E: Control 1, F: Control 2, G: N540K, H: G380R. I, show percentage of formed bone. Magnification: 100X, Scale bar: 100µm

Figure 4: Correlation and prediction of in vivo bone formation with in vitro factors. Empirical modelling was carried out to find the combination of in vitro factors that are predictive of ectopic in vivo bone formation. A: in vitro and in vivo measurements clustered according to their pairwise correlations. B: variable importance plot generated by a first partial least square regression (PLSR) model including all in vitro measurements. Only variables with a importance larger than 0.8 (underlined) have been retained for the final PLSR model. C: Results of the final PLSR model showing the predicted versus measured (‘actual’) values (top) for the percent of bone formed by Collagraft™ (left) and Copios™ (right) carriers. The bottom row shows the difference between measured and predicted values. Symbols are defined as follows: □: control 1; ◊: control 2; ∆: N540K; ○: G380R.
osteocalcin in N540K cells. Together, these represent early to fully differentiated osteoblasts [27,28].

Empirical modelling showed that in vivo bone forming capacity can be predicted by the in vitro level of phosphorylation of Erk1/2, metabolic activity, ALP, Coll1 and osteocalcin expression. In the case of N540K, increased levels of these in vitro measures correspond to the elevated in vivo bone forming capacity. Proliferative ability of the cells has previously been determined to strongly correlate to in vivo bone forming capacity of bone marrow stromal cells [29]. Additionally, Coll1 expression was previously correlated to the bone forming capacity of PDCs [12,30]. This together strengthens the predictive model in this study, which indicates that proliferation along with accelerated osteogenic differentiation may be involved with the phenomenon observed with PDCs containing the N540K mutation in FGFR3. We propose that the specific profile shown in the N540K mutant is due to the intracellular location of this mutation, leading to a ligand-binding independent constitutively active receptor, which becomes internalised upon phosphorylation, and consequently a termination of induced signalling.

This study indicates the importance of FGRF3 signalling in peristeal cell dynamics and indicates a possible cause of increased bone density with some mutations. Additionally, we suggest a potential mechanism for peristeal cell proliferation to enhance bone formation for tissue engineering applications.

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