Polyalanine repeat polymorphism in *RUNX2* is associated with site-specific fracture in bones with high content of intramembranous ossification.

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Abstract

Runt related transcription factor 2 (RUNX2) is a key regulator of osteoblast differentiation. Several variations within RUNX2 have been found to be associated with significant changes in BMD, which is a major risk factor for fracture. In this study we report that an 18bp deletion within the polyalanine tract (17A>11A) of RUNX2 is significantly associated with fracture. Carriers of the 11A allele were found to be nearly twice as likely to have sustained fracture. Within the fracture category, there was a significant tendency of 11A carriers to present with fractures of bones of intramembranous origin compared to bones of endochondral origin (p=0.005). In a population of random subjects, the 11A allele was associated with decreased levels of serum collagen cross links (CTx, p=0.01), suggesting decreased bone turnover. The transactivation function of the 11A allele was quantitatively decreased. Interestingly, we found no effect of the 11A allele on BMD at multiple skeletal sites, although these were not the sites where a relationship with fracture was most evident. These findings suggest that the 11A allele is a biologically relevant polymorphism that influences serum CTx and confers enhanced fracture risk in a site-selective manner related to intramembranous bone ossification.
Introduction

In osteoporosis, diagnosis depends largely on measures of bone mineral density (BMD) by using dual energy X-ray absorptiometry (DXA) in combination with other clinical parameters [1,2]. Increased fracture rates are found in patients with lower BMD, although many fractures occur in patients who are not considered osteoporotic. Fracture risk is not fully explained by BMD as factors that are environmental and genetic are also involved [3]. Although the genetic architecture of fracture risk is complex and polygenic, a reasonable candidate gene is RUNX2: the protein RUNX2 is a critical regulatory factor in osteoblast and chondrocyte differentiation and essential for skeletal development [4-7]. RUNX2 is expressed in mesenchymal progenitors and directs the differentiation of cells into osteoblasts and promotes hypertrophy of chondrocytes [4-7]. Osteoblasts secrete an extracellular matrix rich in collagen type I and other non-collagenous proteins that are responsible for the mineralization of bone extracellular matrix by depositing hydroxyapatite [8]. The combined functions of bone extracellular matrix formation and mineralization regulate bone volume and mineral density. RUNX2 associates with a beta subunit (Cbfβ) and localizes to specific nuclear matrix sites, forming transcriptional complexes to either activate or inhibit the transcription of target genes [9]. Such function transactivation assays have been used to correlate severity of RUNX2 mutations with function [10].

Heterozygous mutations in coding and promoter regions of RUNX2 in humans [4-7] cause the skeletal syndrome Cleidocranial Dysplasia (CCD) [11]. CCD develops when one allele of RUNX2 is entirely functional and the other compromised, a state known as haploinsufficiency; indicating that RUNX2 levels are limiting in skeletal development. CCD is characterized by hypoplasia/aplasia of clavicles, persistently open or delayed closure of sutures, Wormian bones,
supernumerary teeth, short stature and other skeletal abnormalities [11]. Certain bones are more affected than others in CCD: notably the clavicle, scapula, face/mandible, pelvis depending on the severity of the RUNX2 mutation [10,11]. During development, particular bones form by either endochondral or intramembranous ossification or a combination of both processes [12]. The bones most affected in CCD, such as scapula, face/mandible, pelvis and clavicle, have a high content of intramembranous ossification [12]. Skeletal changes observed in heterozygous Runx2 knock-out mice displayed abnormalities that are similar to the CCD phenotype confirming RUNX2 haploinsufficiency as the cause of the syndrome [4-6]. Although not reported yet in humans, mouse Runx2 knock-out homozygotes fail to develop osteoblasts and have no mineralized bone. In the case of haploinsufficiency in mice, greater defects are observed in bones with a higher content of intramembranous ossification compared to bones of greater endochondral origin, mirroring the subtly of human CCD. Regardless of the distinction between intramembranous and endochondral routes of formation, a feature of CCD is a characteristic range of bones that are affected in a particular rank order of severity. Studies of Runx2 gene dosage using isoform specific knockout mice have lead to the proposition that Runx2-II (an isoform driven off the P1 promoter) is more important for endochondral bone formation than the Runx2-I isoform (driven of the P2 promoter) [13]. To that point, mice with intact Runx2-I isoform survive with reduced bone density and are able to increase cortical bone over time, with a form of low bone turnover osteopenia [13]. However, mice with defects in the P1 promoter (which is induced in osteoblasts) that generate gene dose effects also mirror the defects in CCD with a more pronounced defects in bones with a high content of intramembranous ossification [14]. Currently, these data taken together suggest that local tissue specific effects occur and that different ossification types (endochondral versus intra-membranous) are differentially affected by either RUNX2 isoform and/or RUNX2 gene dosage. Based on these propositions, we present the hypothesis that genetic variants of RUNX2
with functional effects may manifest in relative differences of intramembranous bone versus endochondral bone. Even if the distinction between intramembranous and endochondral bone ossification is not as great as supposed, functional variants of RUNX2 may present with a rank order of affected bones similar to the rank order of affected status found in CCD. At present, no clinical index exists that measures the relative density of bones of intramembranous and endochondral origin. However, if this hypothesis is correct, then different rates of fracture may be expected in bones of primarily endochondral origin relative to those of more intramembranous origin, according to RUNX2 genotype.

This study reports that a common amino acid sequence polymorphism in exon 2 of the human RUNX2 gene is significantly related to fracture of bones with higher intramembranous ossification content. As these data suggest that the genetic contribution of a particular gene marker to fracture may be anatomically site-selective, according to the developmental process controlling the particular bone’s formation, this hypothesis should alter the manner in which gene association studies analyze the phenotype of fracture.

**Materials and Methods**

**Clinical collections.**

Geelong osteoporosis study (GOS): GOS is a prospective community based epidemiology study with random recruitment as described previously [15,16]. DNA, serum markers and bone densitometry DXA data was available on 822 females. Markers of bone turnover CTx (collagen
telopeptide) and BSAP (bone alkaline phosphatase) were available on 798 subjects. Dual energy x-ray absorptiometry was performed using Lunar DPX-L densitometer and analyzed using Lunar DPX-L software version 1.31. BMD was measured at the spine (L2-L4) in the posterior-anterior projection, proximal femur (femoral neck, Ward’s triangle, trochanter), whole body, ultradistal (UD) and mid-forearm sites. In vivo short-term precision was 0.6% for the spine, 1.6% for the femoral neck, 2.1% for Ward’s triangle, 1.6% for the trochanter, 0.4% for the whole body, 2.1% for the UD-forearm and 1.1% for the mid-forearm sites. Bone mineral apparent density (BMAD) was calculated for the spine (L2-4) as BMC/(area)\(^{1.5}\) according to Carter et al [17]. Vertebral morphometry at baseline and incident vertebral deformity and fracture at other sites were measured.

The GOS random population survey study subjects were derived from recruitment via the electoral roll and thus represents an essentially unbiased population survey. In this GOS random cohort, five year follow up data were available. In that time, some reported fracture while others did not [18]. Furthermore, those fractures resulting from high impact events such as motor vehicle accidents, were excluded. Within the postmenopausal females of the random population sample, a group existed that did not have incident fracture in the five year surveillance period and had reported an absence of adult fracture history at recruitment. This group represents a control no fracture group.

GOS fracture cases (GOS fracture): fracture samples were ascertained over a two year period from sequential fracture cases at two radiology practices servicing a population of 218,000 inhabitants, as described [19]. All fracture case were approached to join the study. DNA was taken from female fracture patients over 35 years of age with fractures of all types. In order to reduce
confounding variables, the present study was limited to post-menopausal females. The International Classification of Diseases, 9th revision (ICD 9) was used to code fractures. For GOS and GOSF, written informed consent was obtained under the procedures of the Declaration of Helsinki, approved by the Barwon Health Human Research Ethics Committee.

South East Queensland bone study (SEQBS): this study provided 980 subjects of which 671 were genotyped successfully. The study is based at Southport in Queensland, Australia. SEQBS study participants were recruited via notices in the local newspaper. DNA was extracted from leukocytes using standard means. The study is comprised of individuals, twins and siblings and the number of siblings range from 2 to 16 per family. All subjects have anthropometric measures and a detailed history was taken of age of menarche, parity, menopausal status, smoking, and other history of illness. Fracture history was obtained through detailed questionnaire including minor or major severity of impact associated with injury, location of fracture and age at fracture. DXA data were not available. In addition, subjects had data on 23 standard serum and urine metabolic measures including alkaline phosphatase. Written informed consent was obtained and procedures were approved by the Griffith University Human Research Ethics Committee, following the guidelines of the Declaration of Helsinki.

Serum measures.

Serum from subjects of GOS and SEQBS were taken after overnight fast and stored at -20C until assayed by enzyme-linked immunoassay for cross linked collagen products (CTx) [20] and bone specific alkaline phosphatase (BSAP) [21], markers of bone turnover.

Detection of alleles in RUNX2 polyQ/polyA repeat sequence.
Primers were: forward 5’-AGC CTG CAG CCC GGC AAA ATG AGC-3’, reverse 5’-GGG TGG TCG GCG ATG ATC TCC ACC ATG-3’. PCR amplified DNA was resolved on 7.5% polyacrylamide enabling accurate detection of deletion genotypes as described [22]. All DNA samples were genotyped twice on two different occasions. Some DNA samples failed to amplify and therefore were not genotyped. There was no apparent pattern to those DNA that failed.

**Plasmids, cell culture and luciferase assay.**

Full length RUNX2 type-I cDNA (pEF-αA) [23] served as a template to construct RUNX2 11A allele cDNA. PCR was used to amplify the RUNX2 11A from a homozygous individual using the primers 5’-TTCACCACCGGACTCCAATCT-3’ for the 5’ side and 5’-CATCTGGTACCTCTCCGAGGGCTACCACCTTGAAGGCCACGCGACAGGTC-3’ for the 3’ side. The reverse primer contained an EcoNI tag facilitating the cloning of the PCR amplified product into the BglIII-EcoNI site of pUC18RUNX2 [24]. The 11A RUNX2 cDNA was confirmed by DNA sequencing and was excised from pUC18 using XbaI restriction digest and cloned into the XbaI site of pEF-Bos. The human osteocalcin promoter vector pOSLUX (590 basepairs of the promoter upstream of fire-fly luciferase) was as described [24]. Other constructs for glutamine variants 16Q and 30Q were described previously [24]. All cell culture materials were from Invitrogen corporation. Transfection experiments and cell culture conditions were as described previously [24]. In summary, cell lines (NIH3T3 and HEK293) were cultured DMEM with 10% fetal bovine serum (v/v), 1% Penicillin-Streptomycin (v/v) in a 5% CO₂ humidified atmosphere at 37°C. Transfections were done using FuGENE6 according to the manufacturer’s protocol (Promega) with cell counts as described previously [21] using the dual luciferase approach with Renilla luciferase vector, pRL-CMV (Promega). Cells were harvested 48 hours post-transfection and assayed using the dual luciferase system [25].
**Statistical Analysis.**

Fisher’s exact test, chi-square test of association and binary logistic regression analysis was used to analyse the allele frequencies between fracture and non-fracture groups. In the GOS random population, analysis of variance (ANOVA) was used to compare subject anthropomorphic measurements and analysis of covariance (ANCOVA) was used to compare BMD between the different RUNX2 genotype groups while adjusting for the covariates age and weight. Fisher’s protected least significant difference test was used for post-hoc comparisons, after a prior significant omnibus ANOVA F-test. ANCOVA was also used to compare the biochemical markers of bone between the RUNX2 genotype groups while adjusting for age and weight. ANOVA was used to compare subject anthropomorphic characteristics and serum markers of osteoarthritis in different RUNX2 genotype groups with post hoc comparisons. Student’s t-tests or ANOVA were used to analyse the quantitative transactivation analysis data. The general linear model was used for exploration of multifactorial relationships and to analyse transfection data with complex treatments. Equality of variance assumptions were met by logarithmic transformation, where necessary and verified using Levene’s test.

**Results**

**Relationship of 11Ala allele to risk of fracture GOS.**

A total of 598 fracture patients were genotyped. RUNX2 alleles were observed at a frequency of 0.93, and 0.07 for 17A and 11A alleles, respectively. On comparing the allele frequencies of 17A and 11Ala between the control no-fracture group with female fracture cases from the same
Geelong area, a significant difference was observed (Table 1). This represented as a increase of the 11Ala allele in the fracture group relative to the non-fracture group. The 11Ala allele frequency was 0.04 in the control non-fracture group and 0.07 in all fracture cases. Control subjects were recruited from the same community (GOS) and had not sustained fracture during the observation interval and had reported no history of adult fracture (n=448 alleles or 224 subjects). When allele frequencies were compared between all fracture cases and controls, the odds ratio for fracture in the 11A carriers was 1.9 (95% CI, 1.4 to 2.5). A significant over representation of 11Ala alleles in the entire fracture cohort (Fisher’s exact test, p=0.006, Table 1) was observed relative to non-fracture controls.

**Relationship of 11Ala allele to risk of fracture SEQBS study.**

In the South East Queensland Bone study (SEQBS), at a different geographical location within the same country, self reported history of fracture prior to the study was available for all 671 genotyped subjects. The allele frequencies were 0.94 and 0.06 for 17Ala and 11Ala alleles respectively. The fracture data was coded as 1 and 0 for the presence or absence of fracture respectively. There were no significant differences in height, age or weight between the fracture and non-fracture groups. 11A carrier status was coded as zero or one. Logistic regression showed that age (p=0.005) and allele status (p=0.036) were significantly related to ever having fractured. Carriers of the 11A allele were significantly more likely to be in the fracture category compared to non-carriers (p=0.036, OR=1.70, 95% CI 1.04-2.83). This effect was driven by menopausal status: within the postmenopausal group (n=291, with 91 having history of fracture) genotype was a significant predictor of fracture (p=0.014, OR = 2.67, 95% CI = 1.20-5.82), while there was no significant relationship in males or premenopausal females. To further elucidate the effect of the 11A allele on fracture, only those subjects that sustained fracture from minor falls were selected.
for the analysis: 11A carriers were again significantly more likely to have sustained fracture (p=0.011, OR=2.02, 95% CI=1.16-3.51). When fractures occurring as a result of minor falls only were analyzed the 11A allele effect was stronger within the postmenopausal group (p=0.006 OR=3.11, 95% CI=1.39-6.79), an effect similar to that seen in the GOS subjects.

Some individuals in SEQBS had a history of multiple fractures; using ordinal regression 11A status was significantly associated with total fracture count (p=0.015) in postmenopausal females only. Once again, this effect was more obvious considering fractures reported to be from minor falls (p=0.009); a total of 17 fractures were observed among 27 carriers of the 11A allele compared to 77 fractures among 248 non-carriers in the postmenopausal group. Different fracture types were recorded: ankle, spine, hip, distal radius, leg and arm without further discrimination of different types of fracture within those categories. The anatomical location of fracture was related to 11A carrier status in postmenopausal females (p=0.003). The same conclusion was obtained if only low impact fractures were considered (p=0.001). The previous analysis was based on classifying subjects using the first fracture event. Multinomial logistic regression to discriminate first fracture site in postmenopausal females showed that 11A allele status was related to ankle fracture (p=0.007) and possibly distal radius fracture (p=0.068). Among 25 postmenopausal women who had multiple fractures there were 12 ankle and 13 distal radius fractures. Subject fractures were recoded to consider if the patient had ever suffered a particular fracture (distal radius, ankle, spine, arm and hip). Multinomial logistic regression was then used to determine if 11A allele status was associated with fracture site. Age, height and weight covariates were not significantly related to fracture location among the postmenopausal group and these variables were excluded from a stepwise logistic regression. 11A status was significantly related to distal radius (p=0.005) and ankle (p=0.014) fracture location in postmenopausal females, but not other sites.
Postmenopausal 11Ala allele carriers were more likely to have fractured distal radius or ankle (p=0.014, OR=4.11, 95% CI=2.64-6.41) when considering fractures caused by minor falls. This effect was not influenced by smoking history or any other variable in the dataset. The odds ratio of any fracture related to 11A allele carrier status from minor falls in females increased progressively with age from 2.94 (over age 50, n=309), to 3.29 (over the age of 60, n=206) and finally to 6.66 for those over 70 years of age (95% CI 1.68 to 26.4, n=97).

The majority of the dataset, especially the postmenopausal females, were singletons although some siblings were present (73 pairs). The influence of siblings in the dataset was tested using random selection of a single sibling for inclusion with all other singletons. This random selection was done 200 times, with computation of the association between 11A carrier status and fracture in the pre and postmenopausal groups. All 200 random selections within the postmenopausal group resulted in significant association between fracture status and presence of 11A allele with p values ranging between 0.03 and 0.003. The median odds ratio was 2.7 and the median Chi square score was 6.67, representing a p value of 0.009. Repeating this analysis considering only fractures that resulted from minor falls resulted in a median odds ratio of 3.2 with p values ranging between 0.01 and 0.001. No significant associations were observed within 200 similar random selections of singletons from the premenopausal group. These data indicate that the significant association of fracture with 11Ala allele status was not influenced by the presence of sibling pairs in the patient collection.

In order to compare SEQBS to the GOS study, where postmenopausal females who had not reported fracture were taken as controls, a subgroup was selected from SEQBS who were female and over the age 55 and who had not reported fracture on questionnaire. Genotype and allele
counts were not significantly different (p=0.14) from this SEQBS no fracture control group to that of GOS no fracture controls, indicating in a second population that the control group reveals a similar distribution of alleles. Comparison between female controls over 55 and fracture cases over 55 showed a significant association of 11A with fracture (Table 1, p=0.0281) with allele distributions similar to GOS. Although these two populations were recruited from geographically separate parts of the same country, the no fracture controls were not significantly different, legitimizing combining the two populations: Table 1 shows a highly significant association of 11A allele with fracture in these combined Australian data (p=0.0006, OR=1.91, 95% CI=1.54-2.37).

**Relationship of RUNX2 11Ala allele to fracture at different bone sites.**

Data from both GOS and SEQBS showed similar association with fracture for the 11Ala allele. Furthermore, the data from the SEQBS suggested a site selectivity of 11A allele and fracture. The GOS fracture study recruited a larger number of fractures and so has a greater capacity to discriminate this effect. A significant increase in the frequency of 11A alleles was observed in distal radius fractures relative to control non fracture cases (OR 2.0 with 95% CI, 1.4 to 2.9, p=0.02), confirming the increased risk of distal radius fracture associated with the 11A allele observed in SEQBS. Combining distal radius and ankle fracture groups from the GOS fracture cohort resulted in significant association (p=0.01) as had been observed in SEQBS.

Although association with ankle and distal radius fracture was confirmed in both GOS and SEQBS studies, the relationship between 11A alleles and fracture may be more complex. The GOS fracture cohort was drawn from a large population and consisted of many fracture types (Table 2) with spine, hip, distal radius, arm and ankle being the most frequent. The frequency of the 11A allele within each particular fracture sub-group was investigated. A single scapular fracture was
combined with clavicle fractures to represent fractures of the pectoral girdle. The allele frequencies for 11A were determined for each anatomical fracture site; and the 11A allele frequency ranged from zero to 0.25. When the 11A allele frequencies were ranked from highest to lowest, a startling outcome was produced (Table 2): the rank order of frequency of 11Ala alleles according to fracture site reproduced almost exactly the sites of the skeleton most affected by the syndrome CCD. The rank order from highest to lowest for 11Ala allele frequency in fracture sub-groups was: face/mandible, ribs, clavicle/scapula, pelvis and metacarpals. These bones are also the most strongly affected in CCD, dependent on the severity of the particular mutation [11].

In CCD, due to haplo-insufficiency of RUNX2, certain bones are more affected than others. Since these bones are known to be related to RUNX2 haploinsufficiency a priori, corrections for multiple comparisons are not required in subsequent sub-groupings, related to this principle. The cases from GOS fracture were sorted according to the site of fracture and those bones related to the more extreme CCD symptoms were combined. Bones defined as “CCD-related” were: ribs, face/mandible, scapula, clavicle, pelvis, metacarpals, phalanges (n=78) compared to all other fracture types (n=520). Within the category of all fracture cases, where the control non-fracture group was no longer relevant, there was a significant preference for fracture at CCD-related bones within 11A carriers (OR 2.3 with 95% CI, 1.8 to 3.0, p=0.02), compared to fracture at non CCD-related bones. When fractures in bones thought to have a high proportion of intra-membranous ossification were considered (scapula, clavicle, sternum and ribs), compared to all other fracture types, a significant association with 11A alleles was observed (OR 2.9 with 95% CI, 2.0 to 4.3, p=0.006). Compared to controls the association was more prominent: 11Ala alleles were associated with fracture of bones derived primarily from intra-membranous ossification compared to non-fracture controls (OR 5.0 with 95% CI, 3.2 to 7.8, p=0.0006). For bones of primarily
endochondral origin, two fracture types were available that provided the sufficient numbers (108 and 65 for spine and hip fracture, respectively) to test the hypothesis that the 11A allele alters the liability for fracture of a bone according to the majority mode of ossification (either primarily intramembranous or endochondral). The presence of 11A allele was significantly related to fracture at bones with a greater content of intramembranous ossification compared with the two types of fracture at bones of endochondral ossification: spine (OR 3.7 with 95% CI, 3.2 to 6.0, $p=0.006$) and hip (OR 5.0 with 95% CI, 2.8 to 8.9, $p=0.004$). Therefore, within fracture patients, 11A RUNX2 allele carriers have a higher likelihood of fracture at certain CCD-related bones, of higher intramembranous ossification, compared to wild type allele-carriers.

**Relationship of RUNX2 11A allele to CTx and BSAP markers.**

Anatomical location specific fracture was related to RUNX2 11A allele status, suggesting possible functional polymorphism. Such polymorphism may be related to other physiological parameters related to bone biology. Among those genotyped, serum carboxy-terminal collagen crosslink (CTx) and bone specific alkaline phosphatase (BSAP) markers of bone remodeling were available for 803 and 799, respectively, of the random population subjects recruited from the GOS. ANCOVA was used to determine if there were any significant differences in CTx and BSAP levels related to 11A allele carrier status. To satisfy the assumption of homogeneity of variances, natural log transformed CTx and BSAP data was used in the analyses. The analysis revealed that carriers of the 11A allele had significantly lower ln(CTx) measures (5.47±0.91 SD, N=91) compared to non-carriers (5.70±0.81 SD, N=712, $p=0.018$). When adjusted for age and weight, 11A carriers retained significantly lower CTx serum levels ($p=0.03$). In contrast, there were no significant differences in ln(BSAP) levels between 11A carriers and non-carriers ($p=0.706$). Adjusting for the significant covariates age and weight had no material effect on the result.
(p=0.715). CTx was not available for SEQBS: BSAP was not related to 11A status in this study (p=0.53).

**Biochemical analysis of 11A RUNX2 expression construct relative to 17A allele.**

Expression vectors in the context of the RUNX2-I isoform were constructed containing either alternative allele of the alanine repeat: 17A or 11A. These were used in a series of biochemical tests to determine if significant differences existed between the isoforms. Notably, immunofluorescence confocal microscopy was used to measure the extent of nuclear localization when transfected into COS7 cells; a cell line chosen due to low endogenous RUNX2 and ease of transfection. Cells were counted and categorized according to whether staining was nuclear, cytoplasmic or nuclear and cytoplasmic using a RUNX2 antibody (Fig 1A). Under identical conditions of transfection there was no significant difference in the extent of nuclear localization of either isoform (p=0.18), where the majority of staining was nuclear. However under the condition of co-transfection of the co-activator protein CBFB, which forms a heterodimer with RUNX2, a significant difference (p=1x10^-8) was observed with less nuclear staining of CBFB in cells transfected with the 11A version of RUNX2 (Fig. 1B). In other words, a biochemical difference was only revealed by the presence of CBFB co-transfection, suggesting a reduced capacity to assist in CBFB nuclear localization. Western blots using extracts from COS7 cells transfected with expression vectors showed equivalent amounts of protein (Fig 1C).

Quantitative transactivation analysis was carried out using a RUNX2 target gene reporter assay. If RUNX2 11A has differential function, it is likely to be within the physiological range. Therefore an adequately powered transfection experiment is required to detect such a change. Based on power calculations, the experimental plan had 9 replicate transfections for each condition. Two
different RUNX2 responsive reporter plasmids were used: p3RRE, containing three multimerized RUNX2 consensus elements upstream of the firefly luciferase gene and pOSLUC, a human osteocalcin construct driving firefly luciferase [24]. As a measure of transfection efficiency, a construct driving Renilla luciferase was used. We had previously established optimal parameters for RUNX2 responsive transfections and that the Renilla luciferase construct was not sensitive to RUNX2. Empty expression vector (pBOS) was used as an additional control. Human embryonic kidney cell line (HEK293) were used due to low endogenous RUNX2. RUNX2 23Q/11A expression construct was compared with wildtype 23Q/17A and two glutamine repeat variants that we had already established were significantly different from the wild type (16Q/17A and 30Q/17A RUNX2 variants) [24]. In this manner, five constructs and controls were compared against two different responsive reporters, with nine separate transfections per data point (Fig 2). Analysis of variance was used to determine significant differences in luciferase output from reporters for each particular construct. As expected, the previously reported significant decrease in 16Q and 30Q rare RUNX2 variants was verified with 16Q and 30Q constructs being 76% and 69% active (with p=1.2x10^-7 and 3.0x10^-12, compared to 23Q/17A wildtype, respectively). Similarly, compared to wildtype 23Q/17A, the 23Q/11A construct produced significantly lower promoter activity (p=0.008), being 84% active overall. However, the effect depended on the RUNX2 responsive target promoter, being only significantly with the p3RRE reporter construct (77% activity, p=0.0004) and not on pOSLUC (91%, p=0.21).

Notably, a greater difference between RUNX2 17A and 11A isoforms was also observed on the truncated p147 mouse osteocalcin promoter, transfected into NIH3T3 fibroblast cells, where the RUNX2 11A isoform had lower transactivation capacity in the absence of CBFB (p=0.001, Fig 3). Co-transfection of CBFB partner protein essentially normalized the effect to the level found in the
17A wild type isoform (p=0.3). These data support the hypothesis that the 11A variant of RUNX2 is slightly decreased in potential transcriptional activation of target promoters and this effect varies according to the particular target promoter examined and the presence of cofactor proteins such as CBFB.

**Effect of vitamin D receptor on RUNX2 variants in transfection.**

Considering that CBFB appeared to modulate the effect of the RUNX2 11A allelic isoform in the expression construct relative to 17A, we explored the relationship by transfection with another partner protein of RUNX2, the vitamin D receptor (VDR) using the human osteocalcin promoter driving luciferase (pOSLUC). An adequate experiment was designed based on power considerations and NIH3T3 mouse fibroblast-like cells were used. This experiment compared simultaneously two constructs (RUNX2 17A and 11A) at three concentrations of transfected construct (control zero, 15, 30 and 45 ng transfected), with or without transfected VDR (10ng) and with or without treatment with 1,25(OH)$_2$D$_3$ (at 10$^{-7}$ M) with appropriate vehicle and empty vector controls, all as duplicate transfections [24]. The entire experiment was repeated on four separate occasions. All data were pooled and analyzed using the general linear model taking into account all variables. VDR transfection did not have an effect on the target pOSLUC in the absence of 1,25(OH)$_2$D$_3$: induction by 1,25(OH)$_2$D$_3$ was around three-fold as expected. Treatment with 1,25(OH)$_2$D$_3$ in the absence of transfected VDR resulted in significant induction (1.6 fold, p=3.9x10$^{-7}$) presumably from endogenous VDR. The overall general linear model indicated that VDR transfection interacted with 1,25(OH)$_2$D$_3$ treatment (p=5.6x10$^{-4}$) as expected. The activity of pOSLUC was linearly sensitive to the amount of transfected RUNX2 construct as expected (p=4.3x10$^{-11}$). There was a significant difference between RUNX2 17A and 11A vectors (p=1.1x10$^{-6}$) and significant main effects of VDR transfection (p=3.4x10$^{-6}$) and 1,25(OH)$_2$D$_3$
treatment \((p=1.2 \times 10^{-14})\). These observations indicate that the reporter plasmid pOSLUC and the expression constructs were behaving as expected. The RUNX2 11A construct was significantly reduced in capacity to drive the human osteocalcin promoter (pOSLUC) in NIH3T3 cells, in contrast to the lack of an effect seen for pOSLUC in HEK293 cells (Fig. 2). The difference between RUNX2 11A and 17A construct activity (Fig 4) was significant in the: (i) absence of both VDR and 1,25(OH)\(_2\)D\(_3\) \((p=0.01)\); (ii) absence of VDR and presence of 1,25(OH)\(_2\)D\(_3\) \((p=0.001)\); (iii) presence of VDR but absence of 1,25(OH)\(_2\)D\(_3\) \((p=0.013)\). No significant difference was observed in the presence of VDR transfection and treatment with 1,25(OH)\(_2\)D\(_3\) \((p=0.24)\), suggesting that activated VDR drives the promoter to an extent that covers any potential difference between RUNX2 17A and 11A. Because of the decreased basal activity (Fig. 4) of the RUNX2 11A construct driving pOSLUC there appears to be a greater response to 1,25(OH)\(_2\)D\(_3\) than the wild type 17A construct (5.8 fold for 11A versus 3.1 fold for 17A). This means that in the presence of VDR and 1,25(OH)\(_2\)D\(_3\) treatment, the difference between RUNX2 11A and 17A constructs is reduced and no longer significant since VDR induction occurs in the presence of both RUNX2 11A and 17A.

**BMD relationship.**

The effect of the alanine deletion polymorphism on BMD was investigated in the GOS random population subjects. BMD information was available from seven skeletal sites and a total of 822 subjects with genotype: all individuals were included in analysis. There were no significant differences in age, height and weight between the RUNX2 genotype groups. The 11A allele did not show a significant association with BMD at any of the skeletal sites, in keeping with a prior publication on a smaller set of the same cohort [22].
Discussion

We previously presented data supporting a hypothesis that glutamine repeat variants of RUNX2 are associated with lower bone density at a number of skeletal sites and with significantly lower transactivation potential [24]. Although the glutamine variants are rather rare (around 4 in one thousand individuals) a relationship with fracture was established. This prompted us to examine the more frequent 11A polymorphism of the same region of RUNX2. To expand our prior studies of the GOS group [22], in this current work we doubled the sample of GOS random population subjects that were genotyped and completed the genotype of all available fracture cases in the GOS fracture study. In addition, we show the same relationship with fracture, \emph{per se}, in a second independent cohort. In the GOS fracture cohort, we found a site-specific relationship of the 11A allele with fracture that was particular to bones with a greater content of intramembranous ossification. A relationship with BMD was not found, although a significant relationship with serum CTx in the GOS random cohort is evidence for a physiological relationship at the level of bone, since CTx is a marker of bone turnover [20].

How can a relationship with fracture occur in the absence of a relationship with BMD? A simple explanation is that RUNX2 11A may have an effect on BMD at bone sites that were not measured. Since we found fracture-site selectivity and those sites are usually not measured with clinical DXA, this simple explanation is possible. The fact that we observed a significant relationship with fracture at the distal radius, but did not observe a relationship with BMD at this site suggests a non-BMD-related mechanism should be explored. It is possible that the increased risk of fracture associated with the 11A allele is not explained by changes in BMD, but relates to the process of ossification and some unmeasured architectural parameter in bone. Since the bone sites where the
RUNX2 11A allele is associated with fracture have a higher content of intramembranous ossification, the relationship of 11A to bone density and structure needs to be investigated in further studies, with that hypothesis in mind. Aspects of RUNX2 function in bone may influence an individual’s susceptibility to fracture by altering components of bone quality that are independent of bone density as measured by DXA. Alternatively, the effect on BMD occasioned by the 11A variant may be small enough to escape detection in this study. While site selectivity was shown with reasonable numbers in two studies at the distal radius and ankle, a limitation of the study is that smaller numbers of fractures in other bones were available. Despite this, a simple classification system for the skeletal based on those bones more affected in CCD produced a statistically significant outcome, with an odds ratio of nearly three for fracture within bones of high intramembranous content versus high endochondral content for carriers of the 11A allele.

The 11A polymorphism is puzzling in that it is a protein coding variant of an important transcription factor that exists at reasonable frequency in the human population. Examination of primate sequences indicates that the 17A allele is conserved and presumably ancestral: Gorilla (22Q/17A) and chimpanzee (25Q/17A) are different from human in the glutamine repeat, while orangutan (23Q/17A) is identical to human. The Galago (bushbaby, Otolemur crassicaudatus), which is evolutionarily very distant from humans, has 17 alanines (21Q/17A). Of 10 sequenced species available, the only primate RUNX2 that is not 17A is the mouse lemur (Microcebus murinus) with 16A. This indicates remarkable stability of this sequence through time and reaffirms the peculiarity of the 11A allele, which is unique to humans. Currently the 23Q/11A allele has been identified only in Caucasians: frequencies in different ethnic groups are unavailable. Given the conservation of 17A in the repeat over evolutionary time, the possible function of the 11A allele is intriguing. In the comparison between Neanderthal and modern human DNA sequences,
the RUNX2 gene was indicated as highly subjected to genetic selective sweep, indicating evolutionary selection across this locus [26,27]. RUNX2 is therefore a candidate gene for differences between Neanderthal and modern human and a number of different functional changes may be expected within RUNX2. The one Neanderthal sequence available at present is consistent with 17A in the repeat (see genome.ucsd.edu). Notably different skeletal regions between Neanderthal and modern human include ribs and mandible, sites with high intramembranous content. How do our data relate to these points? The RUNX2 11A allele may represent a new allelic variant, specifically related to intramembranous versus endochondral ossification, that is specific to modern humans.

Bones ossify by combinations of the processes of endochondral and intramembranous formation: any particular bone will have a differential input of these two types of mineralization [12,28]. This extent of overlap in endochondral and intramembranous ossification is still a subject of debate in developmental anatomy: for instance the bones of the pelvic and shoulder girdles form by a combination of intramembranous and endochondral ossification. The clavicle has two intramembranous sites and the scapula originates as intramembranous bone then completes through endochondral ossification. In particular, the extent to which ossification at a particular bone is either endochondral or intramembranous in character may be an important factor in understanding the relationship of genes to bone and bone fracture as a trait. Although overly simplistic for the developmental biology of a complex bone element, it is a convenient shorthand to classify bones into endochondral or intramembranous categories. The association of RUNX2 11A allele with fracture derived from higher than expected fracture rates in those bones of higher intramembranous ossification content: so called intramembranous bones. The same assemblage of bones is most affected in CCD, where RUNX2 levels are inadequate in certain tissues due to
haploinsufficiency resulting in a critical threshold effect for abnormality [29]. CCD has a greater impact on those bones with a greater content of intramembranous bone ossification, suggesting that such bones are more sensitive to RUNX2 activity levels than bones with a greater degree of endochondral ossification. Therefore it seems likely that the mechanism of RUNX2 11A allele should relate to effective levels of RUNX2. This was supported by our transfection studies that showed RUNX2 11A was less effective at transcriptional activation through a transfection assay. Although this was a quantitative effect, it was consistent with expectation and was matched with differential measures of nuclear binding. An alternative explanation 11A is not causative but rather is in linkage disequilibrium with another unknown variant that is responsible for the molecular mechanism.

Recent whole genome association analysis shows significant association of BMD to the RUNX2 genomic region [30]. Earlier studies indicated association of RUNX2 alleles with bone phenotypes, BMD and fracture in a number of different populations [22, 24, 31-37]. A RUNX2 P2 promoter haplotype with differential promoter activity in artificial constructs was associated with BMD in Australians [31], two separate Spanish cohorts [32, 33], Russians [34] and Koreans [35]. The P2 promoter drives RUNX2-I isoform, which has been associated with intramembranous ossification in mouse studies [38]. Differential activity of the RUNX2 P2 promoter, changing the relative amount of the RUNX2-1 isoform might be an attractive hypothesis to explain differential fracture at bones of intramembranous ossification. We reported previously that the 11A allele arose in the common ancestral P2 promoter haplotype [31]. Therefore, despite physical proximity to known P2 markers, promoter polymorphism is currently not a good explanation of the association of 11A alleles with fracture, although more studies are required on this topic. Other evidence of clinically relevant functional variation in RUNX2 include association with bone length
in hand and femur [34, 36]. Interesting data in other species on functional variants of the RUNX2 QA repeat include association with skull shape specifically in dogs [39] and snout length more generally in Carnivora [40]. Although this effect on was not generalized across all species of mammal [41] it was also significant in bill length within species of the family Scolopacidae shorebirds [42]. Taken together, these data suggest RUNX2 as a locus of rich genetic variation related to skeletal evolution and a source of clinically relevant variation in humans.

There are limitations to this study. The quantitative effect that we identified by careful transfection studies is lower than might be expected given the relationship to fracture, although only artificial target genes were investigated. Although we did detect significant alterations in RUNX2 activity, these were entirely in vitro assays in immortal cell lines and involve transfections with expression vectors and synthetic targets. There may be other target genes that show greater differential activity or indeed, no differential activity. Furthermore, this alteration in transactivation function may not be related to the fracture phenotype; it is possible that some other biochemical effect is occurring, specific to intramembranous ossification. RUNX2 levels clearly affect intramembranous more than endochondral ossification with a threshold effect for the CCD phenotype at around 80% activity, based on mouse studies [29]. Independent mouse studies verify a dose effect of Runx2 quantity with osteoblast function although the molecular basis of this effect is still under investigation [38,43]. Similarly the BMD measures were mostly not in the bones of interest with respect to fracture and no significant relationship with BMD was found. Although it has already been confirmed by others that RUNX2 is associated with BMD and fracture at the whole genome level (which entailed correction for multiple comparisons involving all measured markers) the levels of significance related to fracture reported here are not significant at the whole genome level. Given the number of possible skeletal sites that can fracture, statistical correction
for multiple fracture locations in whole genome genetic studies would seem impractical and currently not firmly based on theoretical power analysis. Although we do not present corrected p values, considering only two types of bone (such as either high intramembranous or endochondral ossification) means that the p values presented in this study would remain significant after such correction. Although bones with primarily intramembranous ossification are more severely affected in CCD such as shoulder and pelvic girdles [9], no agreed rank order of affected sites is available for the syndrome. The sub-categorization of fracture types in this study was done by simple ranking of allele frequency and a noting a relationship similar to that of CCD skeletal locations. The identification of fracture cases from the GOS fracture study represents a relatively unbiased catchment from a population of over 218,000 inhabitants. Within the fracture category, a significant site specific preference for wrist compared to hip fracture was observed. This resulted from the fact that those fracture types are more abundant than those that are more informative for 11A association such as shoulder and pelvic girdle; this association was not dependent on a control non-fracture group, since it was within the category of fracture. The control non-fracture group was selected from the GOS random population sample as post-menopausal females with both no incident fracture in an observation period and who reported no adult fracture history at recruitment. This control group exhibited a relative depleation of the 11A allele. While the GOS fracture group had clinical assessments of participants, the SEQBS relied on detailed fracture history, similar to that obtained at recruitment for the GOS random sample. Despite the differences in methodology, the SEQBS and GOS yielded similar odds ratios for overall fracture and the control non-fracture groups in both studies had similar allele frequencies.

In conclusion, the 11A allele of RUNX2 is a frequent polymorphism (5% of alleles) which was related to increased risk of fracture in a site specific manner in two independent studies. In
particular, the 11A allele predisposes to fracture of those bones where a greater content of intramembranous ossification occurs, in a manner reminiscent of the syndrome CCD. The 11A deletion allele was seen to have a significant effect on CTx in the GOS random population subjects but not BSAP. A direct effect of the deletion polymorphism on the transactivation ability of RUNX2 was observed; however the effect was weak in the systems analyzed. In vitro transfection studies show that the minor difference between RUNX2 17A and 11A allelic forms is overcome by activated VDR, suggesting the possibility of new clinical studies on the interaction between RUNX2 genotype, vitamin D status and the outcome variable of the fracture locus. These findings suggest that the 11A deletion is a biologically significant polymorphism altering bone parameters and conferring an increased risk of fracture in a site selective manner. Further to this point, we suggest that future whole genome analysis of fracture take into account fracture subtypes based on categories of intramembranous bone ossification.

**Acknowledgments**

Author contributions: This work was conceived by NAM, who also genotyped samples, did statistical analysis and takes responsibility for the study; further genotyping and plasmid vectors were made by AS; fracture samples and participant data were collected by LRG, JP and GCN; transfection and cell biology experiments were done by AS, MO, NY, NF and PP; GSS provided reagents and expertise; the manuscript was written by NAM. Professor Mark Forward is thanked for critical review of the manuscript and stimulating discussion. These studies were supported by an Australian National Health and Medical Research Council grant to NAM, PP, GSS and GCN.
References


Table 1. Genotype counts, 11A allele frequency ($f_{11A}$) in non-fracture controls versus all fracture cases in females from two studies (GOS and SEQBS) and those studies combined.

<table>
<thead>
<tr>
<th>Study</th>
<th>17A/17A</th>
<th>17A/11A</th>
<th>11A/11A</th>
<th>$f_{11A}$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>207</td>
<td>17</td>
<td>0</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Fracture</td>
<td>518</td>
<td>78</td>
<td>2</td>
<td>0.07</td>
<td>0.006</td>
</tr>
<tr>
<td>SEQBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161</td>
<td>11</td>
<td>1</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Fracture</td>
<td>65</td>
<td>10</td>
<td>1</td>
<td>0.08</td>
<td>0.0281</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>368</td>
<td>28</td>
<td>1</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Fracture</td>
<td>583</td>
<td>88</td>
<td>3</td>
<td>0.07</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

GOS control is described in the methods, GOS fracture comes from the fracture study, SEQBS data shown is for females over the age of 55 from the random population cohort. GOS and SEQBS controls were not significantly different (p=0.14) and were combined. P value is Fisher exact test.
Table 2. Genotype counts, 11A allele frequency \( fr(11A) \) and Fisher’s exact test p value according to anatomical locations ranked by the frequency of the 11A allele in the fracture group.

<table>
<thead>
<tr>
<th>Fracture group</th>
<th>17A/17A</th>
<th>17A/11A</th>
<th>11A/11A</th>
<th>( fr(11A) )</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-fRACTure</td>
<td>518</td>
<td>78</td>
<td>2</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Patella</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.682</td>
</tr>
<tr>
<td>Upper leg</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>0.545</td>
</tr>
<tr>
<td>Foot</td>
<td>30</td>
<td>2</td>
<td>0</td>
<td>0.03</td>
<td>0.280</td>
</tr>
<tr>
<td>Hip</td>
<td>60</td>
<td>5</td>
<td>0</td>
<td>0.04</td>
<td>0.203</td>
</tr>
<tr>
<td>Toe</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0.04</td>
<td>0.385</td>
</tr>
<tr>
<td>Spine</td>
<td>97</td>
<td>11</td>
<td>0</td>
<td>0.05</td>
<td>0.117</td>
</tr>
<tr>
<td>Finger/thumb</td>
<td>17</td>
<td>2</td>
<td>0</td>
<td>0.05</td>
<td>0.268</td>
</tr>
<tr>
<td>Carpals</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0.06</td>
<td>0.351</td>
</tr>
<tr>
<td>Humerus</td>
<td>33</td>
<td>5</td>
<td>0</td>
<td>0.07</td>
<td>0.118</td>
</tr>
<tr>
<td>Forearm</td>
<td>42</td>
<td>5</td>
<td>1</td>
<td>0.07</td>
<td>0.067</td>
</tr>
<tr>
<td>Ankle</td>
<td>40</td>
<td>7</td>
<td>0</td>
<td>0.07</td>
<td>0.063</td>
</tr>
<tr>
<td>Distal radius</td>
<td>91</td>
<td>16</td>
<td>0</td>
<td>0.07</td>
<td>0.020*</td>
</tr>
<tr>
<td>Lower leg</td>
<td>35</td>
<td>7</td>
<td>0</td>
<td>0.08</td>
<td>0.044</td>
</tr>
<tr>
<td>Metacarpals</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>0.11</td>
<td>0.132</td>
</tr>
<tr>
<td>Pelvis</td>
<td>13</td>
<td>4</td>
<td>0</td>
<td>0.12</td>
<td>0.041*</td>
</tr>
<tr>
<td>Ribs, sternum</td>
<td>16</td>
<td>5</td>
<td>1</td>
<td>0.16</td>
<td>0.003*</td>
</tr>
<tr>
<td>Scapula/clavicle</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0.20</td>
<td>0.054</td>
</tr>
<tr>
<td>Face/mandible</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0.25</td>
<td>0.011*</td>
</tr>
</tbody>
</table>

1 p value is the Fisher’s exact test hypergeometric probability based on a comparison of allele counts between control no-fracture group and the fracture subgroup. * denotes significant effect.
Table 3. Allelic association of 11A allele with fracture at bones with intramembranous ossification (scapula, clavicle, ribs, sternum) in comparison to those with a larger content of endochondral ossification (Hip and Spine). Allele counts, allele frequency \(fr(11A)\) and probability (p value) by Fisher’s exact test.

<table>
<thead>
<tr>
<th>Fracture bone type</th>
<th>17A</th>
<th>11A</th>
<th>(fr(11A))</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intramembranous</td>
<td>45</td>
<td>7</td>
<td>0.134</td>
<td></td>
</tr>
<tr>
<td>Hip</td>
<td>125</td>
<td>5</td>
<td>0.038</td>
<td>0.004</td>
</tr>
<tr>
<td>Spine</td>
<td>205</td>
<td>11</td>
<td>0.051</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Figure 1. RUNX2 17A and 11A constructs have differential nuclear residence. A. Confocal immunofluorescence labeled with anti-RUNX2 antibody to detect nuclear or cytoplasmic residence. A comparison of RUNX2 17A and 11A reveals small differences in RUNX2 nuclear residence. B. The ability of RUNX2 to enhance the nuclear localization of the partner protein CBFB is diminished in RUNX2 11A isoform. White numbers indicate percentages of cells with that particular type of staining. C. Western blot indicates that the expression of the 23Q/11A variant in transfected cells does not appear to differ from that of the wild type 23Q/17A variant. Mock indicates mock transfected cells. Tracks were run on the same gel and the image cut to remove intervening tracks. The intervening tracks and controls appear in Morrison et al [24].

Figure 2. RUNX2 17A and 11A constructs have differential biochemical behavior. A. Transfection comparison of the activities of RUNX2 expression constructs driving two different reporter plasmids (p3RRE and pOSLUC) in HEK293 cells. pBOS is empty vector control for baseline promoter activity of the RUNX2 responsive plasmids. 23Q/17A construct (17A) has higher activity in both assays than the RUNX2 23Q/11A variant allele (11A). On the reporter (p3RRE) the RUNX2 23Q/11A variant was significantly different from the wild type 23Q/17A allele (p=0.0004) and was similar in expression to rare allelic variants of the glutamine repeat (16Q/17A and 30Q/17A, labeled as 16Q and 30Q, respectively). In contrast, on the pOSLUC promoter the RUNX2 11A variant was not significantly different from wild type.
Figure 3. Effect of CBFB cotransfection on the comparison between RUNX2 17A and 11A constructs on a truncated mouse osteocalcin promoter, p147. A difference is observed between RUNX2 17A and 11A constructs in the absence of CBFB. In the presence of CBFB, the p147 target promoter activity is induced and the difference between RUNX2 17A and 11A constructs is eliminated.

Figure 4. Effect of VDR transfection on difference between RUNX2 17A and 11A constructs in transfection. Constructs were transfected at four concentrations (1, 15, 30 and 45 ng) with or without VDR (10ng) and with or without treatment with 1,25(OH)2D3 (1,25D3 in figure) at 10^{-7}M. Data presented are the marginal means derived from the general linear model and adjusted by linear regression to 12.5 ng of transfected vector. Error bars are one standard error of the mean. Promoter activity is the control Renilla adjusted pOSLUC firefly luciferase activity. In all cases within a treatment, is the comparison between RUNX2 17A and 11A significantly different excepting the single case marked where cells were transfected with VDR and treated with 1,25(OH)2D3.
Fig 1
Fig 2.
Fig 3.
Fig 4.