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European Journal of Cancer (2012) **48**, 3429–3438

Available at www.sciencedirect.com

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The activities of Smad and Gli mediated signalling pathways in high-grade conventional osteosarcoma ☆

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Available online 4 August 2012

KEYWORDS
Osteogenesis
Metastasis
Migration
Angiogenesis
Bone neoplasm

Abstract High-grade conventional osteosarcoma is a malignant tumour predominantly affecting adolescents and, despite multimodal intensive therapy, lethal for one third of the patients. Although there is currently detailed knowledge of normal skeletal development, this has not been integrated into research on the genesis of osteosarcoma. Recently we showed that the canonical Wnt pathway is not active in osteosarcoma and that its reactivation is disadvantageous to osteosarcoma cells. Since Wnt is regulating normal skeletogenesis together with other pathways, here we report on the activities of the bone morphogenic protein (BMP), the transforming growth factor beta (TGFβ) and the hedgehog (Hh) pathways in osteosarcoma. Human osteosarcoma samples ($n = 210$), benign bone tumours of osteoblastic lineage called osteoblastoma ($n = 25$) and osteosarcoma cell lines ($n = 19$) were examined. For pathway activity luciferase transcriptional reporter assays and gene and protein expression analyses were performed. Immunohistochemical analysis of phosphorylated Smad1 and Smad2, the intracellular effectors of BMP and TGFβ, respectively, showed nuclear expression of both proteins in 70% of the osteosarcoma samples at levels comparable to osteoblastoma. Interestingly cases with lower expression showed significantly worse disease free survival. This may imply that drugs restoring impaired signalling pathways in osteosarcoma might change the tumour's aggressive clinical course, however targeted pathway modulation *in vitro* did not affect cell proliferation.

☆ *Support:* This work was financially supported by the Dutch Cancer Society (KWF) [Grant 2009-4012] and by EuroBoNet, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumours [Grant LSHC-CT-2006-018814].
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0959-8049
http://dx.doi.org/10.1016/j.ejca.2012.06.018

1. Introduction

High-grade conventional osteosarcoma is an aggressive malignant bone tumour preferentially found in young adults.[1] The 5-year survival rate of osteosarcoma patients has improved up to about 60%,[2] however for the patients who do not respond to the presently applied chemotherapy alternative treatment regimens are required. Most conventional osteosarcomas are located at the metaphysis of the long bones adjacent to the growth plate where elongation of the bones, especially at puberty, is most active. Therefore we hypothesised that physiological regulation of signal transduction involved in normal skeletal differentiation is impaired in osteosarcoma. Restoring the differentiation might change the aggressive osteosarcoma into a less aggressive, more differentiated tumour.[3]

Bone is continuously remodelled by a balanced process of bone formation by osteoblasts and bone resorption by osteoclasts. During this process several major signalling pathways such as canonical wingless (Wnt), bone morphogenic protein (BMP), transforming growth factor beta (TGFβ) and hedgehog (Hh) regulate the differentiation of mesenchymal stem cells (MSCs). This indicates that alteration of one or more of these pathways could affect the normal differentiation and might cause disease.[4–6] Previously we showed that in contrary to other reports[7] the Wnt pathway is inactive in osteosarcoma and that its reactivation can inhibit osteosarcoma cell proliferation and stimulate differentiation.[8] TGFβ is found to be abundantly present in normal bone matrix[9] and the pathway plays a prominent role in many different tumour types.[10] As a result of BMP and TGFβ activity, Smad1 and Smad2 respectively become phosphorylated and are translocated to the nucleus where they activate specific genes.[11] In human bone, the hedgehog pathway (Hh) tightly regulates growth and differentiation.[12,13] Hh starts a cascade which results in stabilising *GLI*, which subsequently activates downstream target genes, especially itself and the Hh repressor *PTCH*.

In this study we investigated the activities of the BMP/TGFβ and Hh pathways in osteosarcoma. To consider the relation between impaired differentiation and malignant transformation we compared the activity levels in osteosarcoma to those found in osteoblastoma. The latter is a benign bone tumour with fully terminal osteoblastic differentiation that interestingly never progress to a malignant phenotype. In addition, the effect of pathway modulation on osteosarcoma cells was assayed by investigating cell proliferation and migration. We show that the TGFβ/BMP pathways are active in most osteosarcomas and that a low activity of TGFβ signalling significantly predicts poor survival of the patients. Furthermore the Hh pathway shows various activity levels in osteosarcoma and its inhibition by cyclopamine inhibits proliferation of osteosarcoma cells. However this is independent of Hh activity and could therefore be considered as an off-target effect.

2. Materials and methods

2.1. Clinical samples

Formalin-fixed, paraffin-embedded (FFPE) tissues were retrospectively collected from 127 patients diagnosed with high-grade primary osteosarcoma selected for osteosarcoma trials (according to EURAMOS1 and EORTC Protocol Nos 80831, 80861 and 80931), with a total of 210 samples. In addition FFPE tissues from 25 osteoblastomas were collected. Histological details and clinical follow up data are depicted in Supplementary Table 1. All tissue samples were handled in a coded fashion according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

2.2. Immunohistochemical staining (IHC)

Tissue cores from tumour areas were taken to construct two tissue arrays (TMAs) as previously described.[4] Slides were stained with pSmad1 (1:50 dilution) and pSmad2 (1:200 dilution) antibodies (Cell Signalling, Danvers, United States of America (USA)) and independently evaluated by two observers (P.C.W.H. and C.A.), scoring nuclear staining for their intensity (0 = no, 1 = weak, 2 = moderate, 3 = strong) and the percentage ($1 \leqslant 25\%$, 2 = 25–50%, 3 = 50–75%, $4 \geqslant 75\%$) of positive neoplastic cells. The total score of a sample was calculated as the average sum of intensity and percentage of all its cores, ranging from 0 to 7.

2.3. Genome-wide gene expression profiling and data analysis

Genome-wide expression profiling was performed on pre-treatment diagnostic frozen biopsies of 53 high-grade osteosarcoma patients from the EuroBoNet consortium,[14] of which most were not available on the aforementioned paraffin tissue arrays, and on 12 MSCs cultures. RNA isolation, cDNA synthesis, cRNA amplification, Illumina Human-6 v2.0 Expression BeadChip hybridisation, qPCR validation and microarray data analysis were performed as previously described.[14] MIAME-compliant data have been deposited in the GEO database (www.ncbi.nlm.nih.gov/geo/, accession numbers GSE21257 for biopsies, GSE28974 for MSCs). Pathway analysis was performed on Wnt, BMP, TGFβ and Hh pathways using the Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

2.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA isolation, cDNA reactions and qRT-PCR were performed as previously described.[4] The following primers for *PTCH1* and *GLI1* were used respectively: forward 5′ CCACGACAAAGCCGACTACAT 3′, reverse 5′ GCTGCAGATGGTCCTTACTTTTTC 3′ and forward 5′ TGCAGTAAAGCCTTCAGCAATG 3′, reverse 5′ TTTTCGCAGCGAGCTAGGAT 3′. The CT values were normalised using the $2^{-\Delta\Delta}$CT method.[15]

2.5. Cell culture and reagents

Culturing conditions of the cell lines used are depicted in Supplementary Table 2. The human osteosarcoma cell lines as well as other control cell lines were purchased from ATCC or obtained from EuroBoNet partners.[16] After performing multiple dose–response experiments for all drugs, the following dosages were used to specifically inhibit or stimulate the pathways. TGFβ activity was inhibited by 10 μM SB-431542 (Tocris Bioscience, Bristol, United Kingdom (UK)) and stimulated by 5 ng/ml TGFβ3 (Sigma, Munich, Germany). BMP activity was inhibited by 100 nM LDN-193189 (Stemgent Inc., San Diego, CA, USA) and stimulated by 300 ng/ml BMP4 (R&D systems, Minneapolis, USA). Hh activity was inhibited by 10 μM KAAD-cyclopamine (Toronto Research Chemicals Inc.) and 4 μM arsenic trioxide (Sigma) and stimulated by adding 300 ng/ml recombinant hedgehog (R&D systems). Mouse myoblast cells C2C12, human liver carcinoma cells HepG2 and human pancreatic carcinoma cells PANC1 were used as positive controls for BMP, TGFβ and Hh activities, respectively. Human MSCs were not used as positive controls because of technical difficulties leading to unacceptably low transfection efficiency.

2.6. Plasmids

The BMP-responsive element (BRE)-luciferase construct and the TGFβ pathway responsive plasmid containing a (CAGA)12-luciferase reporter, have been described previously.[17,18] The Hh pathway reporter Gli-luciferase plasmid was a kind gift from Dr. P. Beachy (Stanford, CA, USA).[19] pRL-CAGGS vector containing renilla luciferase under a constitutive CAGGS promoter was used for quantifying the transfection efficiency (Promega, Leiden, The Netherlands). A Gli1-SiRNA-SMART pool (Dharmacon, Chicago, USA) was used to specifically down-regulate the Hh activity by inhibiting *GLI-1*.

2.7. Proliferation assay

The number of viable cells was determined by using a Cell Titer-96 Aqueous One Solution Cell Proliferation Assay (MTS) from Promega according to the manufacturer's instructions and measuring each condition in triplicate.

2.8. Transient transfection and luciferase assay

Cells were seeded at a density of 5000 cells per well in 96-well flat-bottom plates. Next day, 100 μl transfection complex was prepared with 1.95 μg of each reporter plasmid and 0.05 μg of pRL-CAGGS. 5 μl of the mix was added per well using Fugene HD transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. After 24 h the medium was replaced by medium supplemented with drugs as indicated or DMSO as vehicle. After 24 h incubation, cells were harvested and luciferase activity was measured with a Victor 3 Multilabel Counter 1420-042 (Perkin Elmer, MA, USA). The ratio of firefly/renilla fluorescence was calculated to normalise reporter activity. Three independent transfections were performed, each in triplicate.

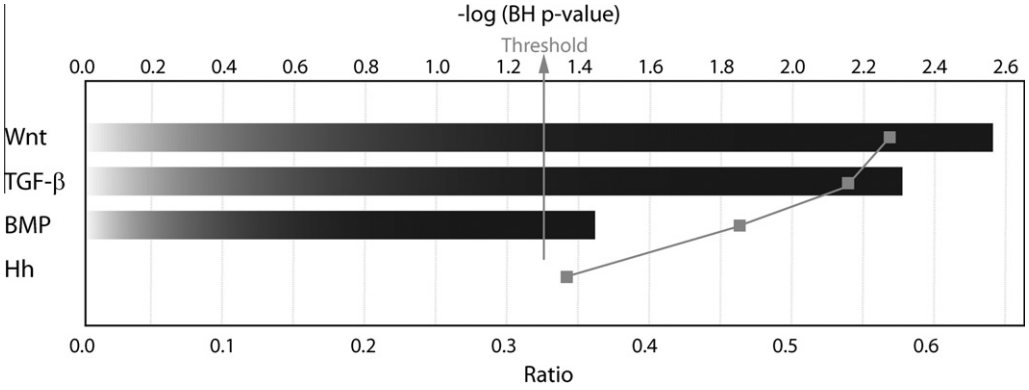

Fig. 1. Pathway analysis. Genome wide gene-expression analysis on a subset of osteosarcoma pre-treatment cases ($n = 53$) shows that the developmental pathways are deregulated in osteosarcoma as compared to mesenchymal stem cells (MSCs) ($n = 12$, bars and upper scale). The ratio is calculated by the number of molecules in a given pathway that meets cut-off criteria (false discovery rate (FDR)-adjusted p-values < 0.05), divided by total number of molecules that are annotated on the microarray and make up that specific pathway (lines and lower scale).

2.9. Migration assay

Cells were cultured in 6-well plates until 90% confluence and starved overnight in 0.5% FCS medium. Next day cells were scratched using a sterile 200 µl pipette tip and medium containing 1.5 ml of each drug was added.

2.10. Statistical analysis

Statistical analyses were performed using SPSS software version 16.0. Results are presented as means ± SEM. A p value of less than 0.05 was considered as significant.

3. Results

3.1. Involvement of developmental pathways in osteosarcoma

Pathway analysis performed by using the Ingenuity Pathway Analysis (IPA) software on a cohort of osteosarcoma pre-treatment cases ($n = 53$) showed that the

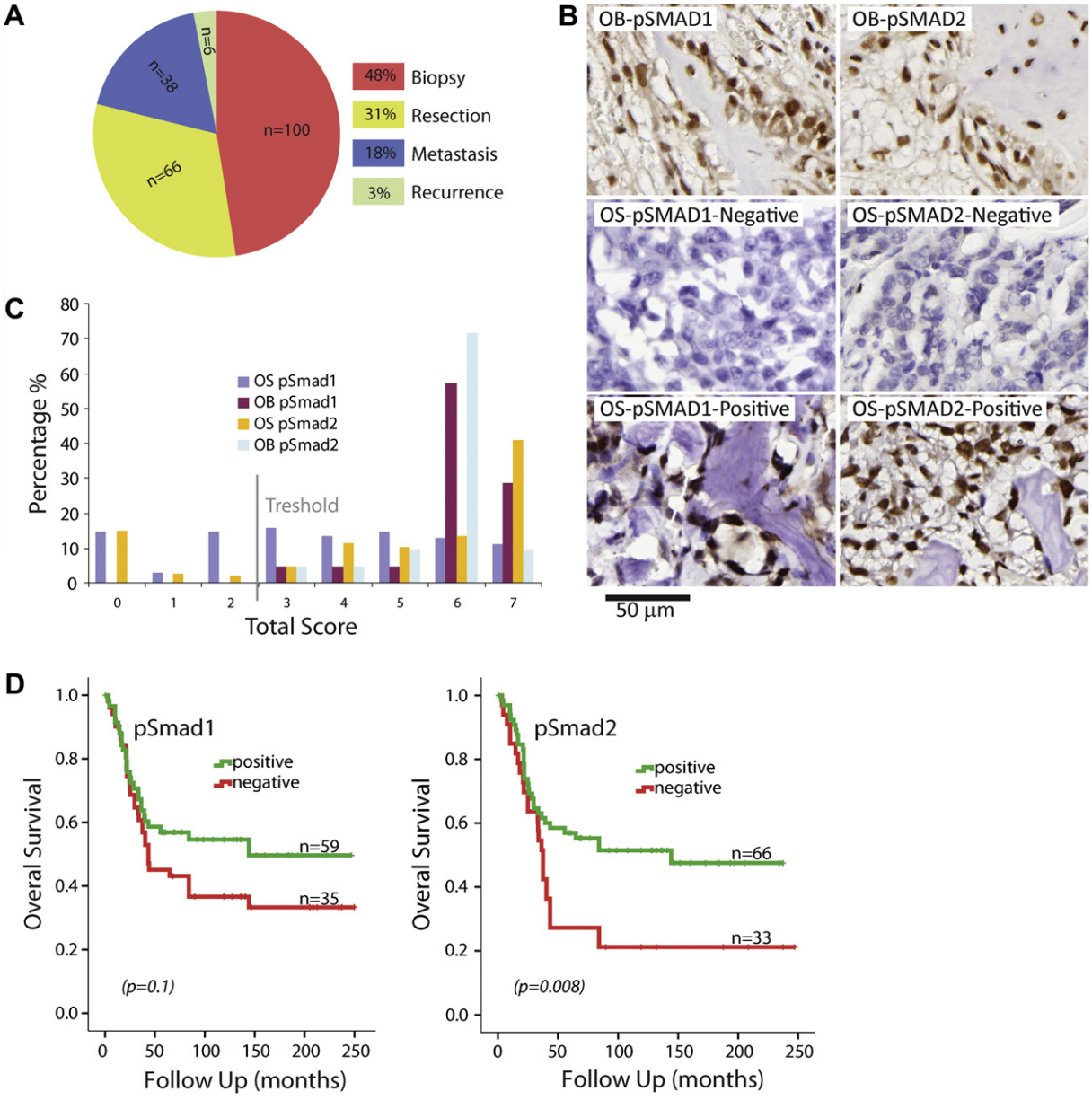

Fig. 2. The bone morphogenic protein (BMP)/the transforming growth factor beta (TGFβ) pathway activities by protein expression analyses. (A) Pie chart showing the sample types of the osteosarcoma tissues used. (B) Representative immunohistochemical staining (IHC) pictures showing that pSMAD1 and pSMAD2 are expressed in all osteoblastoma (OB) cases while in osteosarcoma (OS) a subgroup was found with very low or absent expression. (C) Bar graph depicting the percentages of the scores (0–7) for pSmad1 and pSmad2 protein expression in OS and OB. The lowest score found in OB was 3 for which the threshold of positivity for these proteins was set at 3. (D) Kaplan–Meier survival curves are shown for comparing pre-treatment osteosarcoma cases with low and high (threshold score = 3) nuclear pSmad1 and pSmad2 expression. Cases with low pSmad1 and pSmad2 nuclear expression show worse overall survival which is statistically significant for pSmad2 expression ($p = 0.008$).

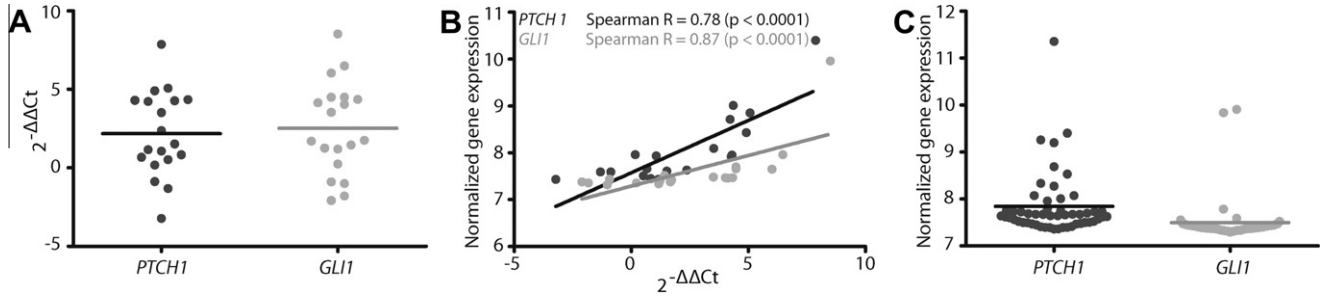

Fig. 3. The hedgehog (Hh) pathway activity by gene expression analysis. (A) Expression levels of the *PTCH1* and *GLI1* genes measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in 19 osteosarcoma cell lines are shown. Levels were normalised by the $2^{-\Delta\Delta Ct}$ method. Each dot represents one cell line indicating a wide variation of expression levels in osteosarcoma cell lines. (B) Spearman's correlation analysis showing significant correlation of *PTCH1* and *GLI1* expression determined by the qRT-PCR and the whole genomic expression array analysis of these 19 cell lines (unpublished data). This validates the expression array results and confirms the wide spread of expression levels of *PTCH1* and *GLI1*. (C) Whole genomic expression array data of the 53 pre-treatment patient samples again show variability of the gene expression levels of the Hh key genes *PTCH1* and *GLI1*.

Wnt, TGFβ and BMP developmental pathways but not Hh are significantly deregulated in osteosarcoma as compared to the presumed progenitor cells of osteosarcoma, i.e. normal MSCs (Fig. 1). Although this analysis does not show whether the pathways are up- or down-regulated as compared to their activities in MSCs, it indicated that Wnt, TGFβ and BMP signalling pathways may be involved in osteosarcoma pathogenesis warranting further investigation of the activity by functional studies.

3.2. BMP/TGFβ pathways are active in most osteosarcoma samples and TGFβ signalling is significantly associated with prognosis

To investigate the activity of the BMP/TGFβ pathways in clinical cases (Fig. 2A), nuclear protein expression of phosphorylated (p) Smad1 (BMP) and pSmad2 (TGFβ) was assayed by IHC as shown in Fig. 2B. pSmad1 and pSmad2 were both expressed in most samples, 85.4% (140/164) and 85.2% (127/149), respectively (Fig. 2C). To investigate whether incomplete osteogenic differentiation would be related to aggressive behaviour, expression levels were compared to those found in osteoblastoma. As both were also highly expressed in osteoblastomas 100% (25/25) a score threshold of 3 (lowest score in osteoblastoma) was set to indicate low activity of the pathways (Fig. 2C). Using this threshold, pre-treatment cases (biopsies) with low pSmad1 and pSmad2 expression showed lower overall survival, $p = 0,1$ and $p = 0008$, respectively (Fig. 2D). For this analysis only pre-treatment samples were used since chemotherapy may affect Smad phosphorylation. The differences in survival only appear after 50 months confirming that patients with metastatic disease at diagnosis have poor survival regardless of the Smad expression.

3.3. Hh pathway activity is highly diverse among osteosarcoma samples and cell lines

The Hh activity, measured by quantifying the gene expression levels of key downstream genes *PTCH1* and *GLI1*, was highly variable among cell lines (Fig. 3A), however the values correlated significantly to the data found by whole genomic expression analysis (Fig. 3B). After this validation the high variability in *PTCH1* and *GLI1* expression in patient samples (Fig. 3C) was considered as evident and did not correlate with clinical behaviour.

3.4. Functional assays confirm the activity of the pathways

The luciferase reporter assays showed functionally active BMP (Fig. 4A) TGFβ (Fig. 4B) and Hh (Fig. 4C) pathways in most cell lines. The OSA cell line showed the highest (intrinsic) activity levels for these three pathways. The MG-63 cell lines did not show any Hh activity and stimulation of the pathway did not increase the activity level (Fig. 4C). Except for this, all three pathways could be either stimulated or inhibited by using the appropriate drugs.

3.5. Pathway modulation does not inhibit proliferation or migration of the cells

Pathway modulation was performed by treating cells transfected with luciferase reporter constructs with the indicated drugs. Functional reactivity of all pathways was confirmed by showing increase or decrease of luciferase activity upon treatment (Fig. 4). Except for cyclopamine, treatments did not significantly affect the proliferation (Fig. 5A and B) or the migration rates of the cells (Fig. 5C). To further investigate whether the

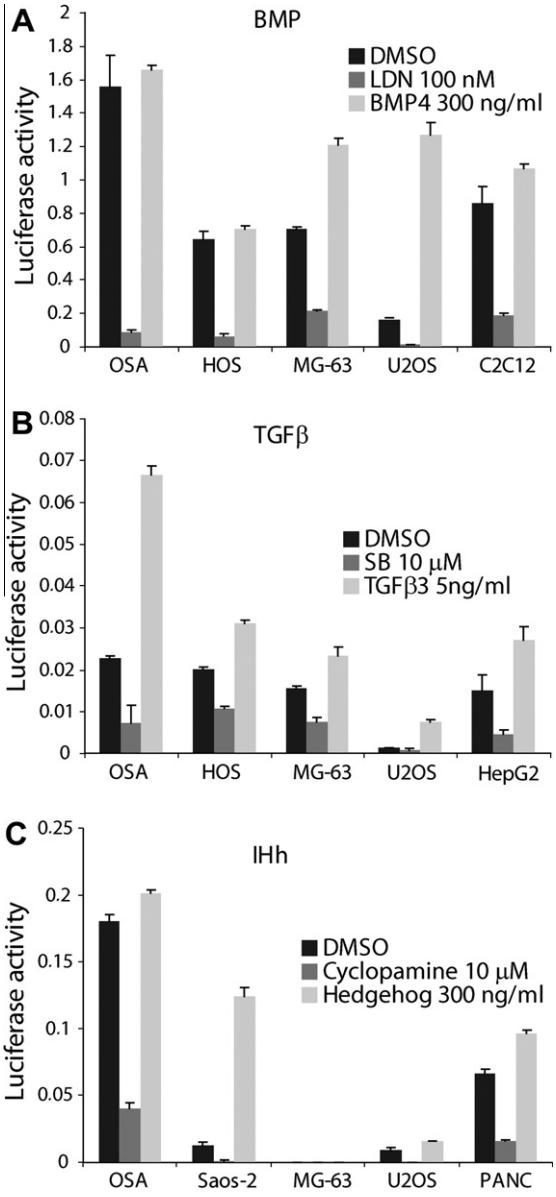

Fig. 4. Functional pathway activity and modulation. (A) Graph depicts bone morphogenic protein (BMP) activity of the osteosarcoma cell lines measured by a luciferase reporter system. In all cell lines pathway activity could be inhibited by adding 100 nM of LDN-193189 and stimulated by using 300 ng/ml of BMP4. (B) As (A) however here the transforming growth factor beta (TGFβ) pathway was inhibited by adding 10 μM SB-431542 and stimulated by adding 5 ng/ml TGFβ3, showing altered activities. (C) The hedgehog (Hh) pathway activity was not found in all osteosarcoma cell lines at levels comparable to the control PANC1 cell line. The OSA cells show high Hh activity consistent with the *GLI1* amplification observed in this cell line and its primary tumour[40] while there is no activity in MG-63 cells. In all other lines the Hh activity was successfully inhibited by the addition of 10 μM cyclopamine and stimulated by adding 300 ng/ml hedgehog.

anti-proliferative effect of cyclopamine was mediated by Hh inhibition, the Hh pathway was more specifically inhibited in OSA cell line – which showed the highest activity of Hh – by using a Gli1-siRNA SMARTpool and arsenic trioxide. The pathway activity significantly

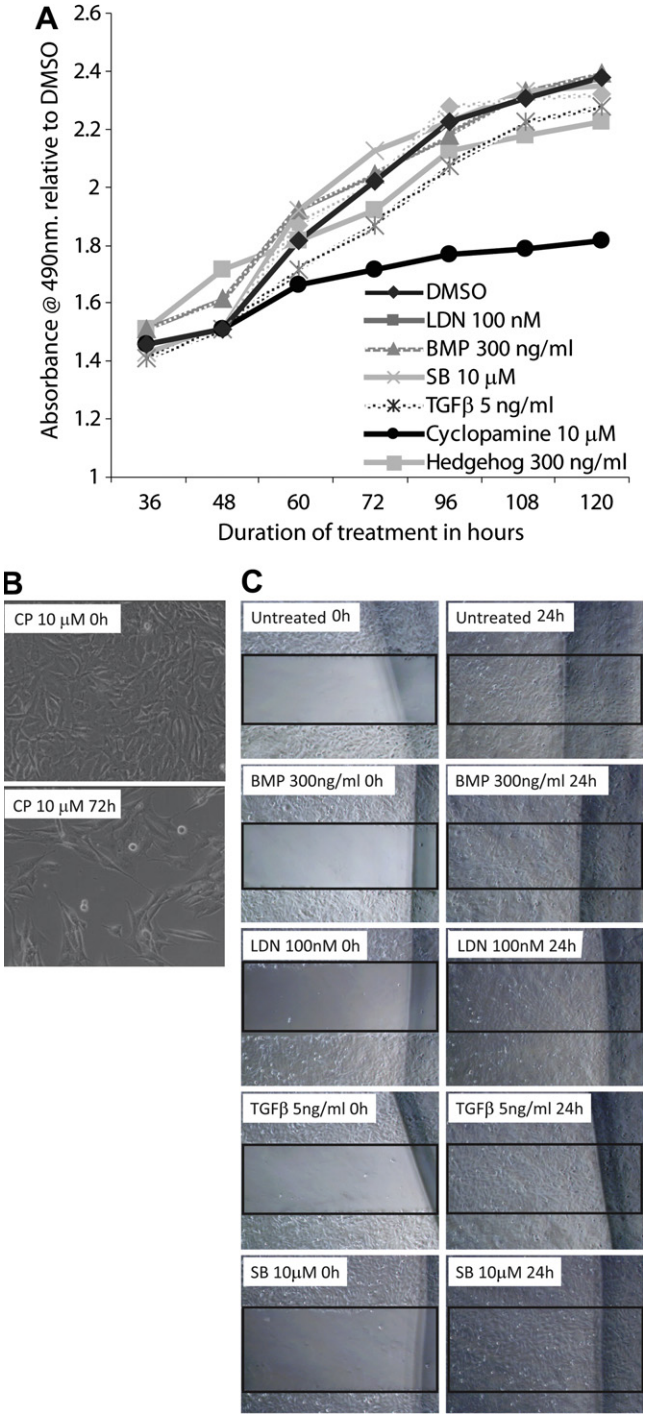

Fig. 5. Effects of pathway modulation on proliferation and migration. (A) Line graphs represent proliferation of the MG-63 cells after pathway modulation by the drugs as indicated or untreated (DMSO). The graph is representative for all cell lines, MG-63 was chosen to show since in this cell line no hedgehog (Hh) activity was present as shown in Fig. 3. Except for cyclopamine there was no significant effect on cell proliferation up to 120 h after treatment. (B) Pictures of the Saos-2 cell line and representative for the other cell lines showing decreased cell numbers after treatment with cyclopamine indicating decreased cell proliferation as shown in (A). (C) Pictures of cell cultures showing that modulation of the bone morphogenic protein (BMP)/the transforming growth factor beta (TGFβ) pathways by the drugs as indicated did not affect the migration of the cells. Pictures were taken from the HOS cell line and are representative for all cell lines.

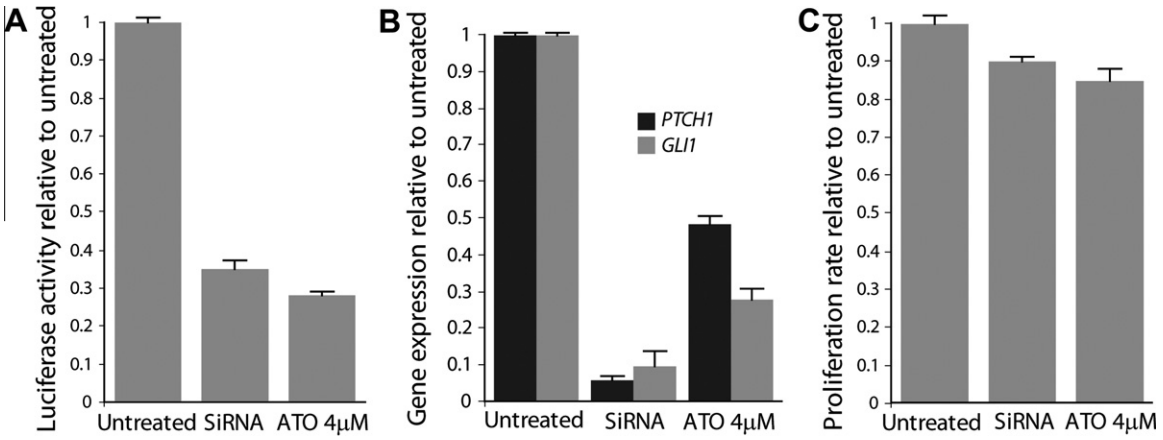

Fig. 6. Specific hedgehog (Hh) repression. Experiments assaying more specific inhibition of the Hh pathway by using a Gli1-SiRNA construct and arsenic trioxide (ATO) in OSA cells which have the highest Hh activity. While the pathway activity was lowered to about 10% confirmed by the luciferase reporter assay (A) and the expression of the transcripts *PTCH1* and *GLI1* (B), there was no effect on the proliferation of the cells (C).

decreased measured by the luciferase reporter (Fig. 6A) and gene expression levels of *PTCH1* and *GLI1* (Fig. 6B), however proliferation was not affected (Fig. 6C).

4. Discussion

Reports from international collaborative studies have shed light into osteosarcoma aetiology and pathogenesis.[4,5,8,14,20] Impaired differentiation of a mutated, genomically instable MSC may contribute to osteosarcoma-genesis.[5,8,21,22] Previously we confirmed this by showing aberrant Wnt pathway activity in osteosarcoma.[8] In this study we analysed other important pathways in bone homeostasis, i.e. the BMP/TGFβ and Hh pathways (Fig. 7).

Next to the essential roles of the BMP signalling in normal skeletal development, studies have indicated its involvement in promoting metastasis.[23,24] However research addressing the functionality of this pathway specifically in osteosarcoma, is limited.[25,26] As BMPs can induce ectopic bone formation[27] and osteosarcoma is characterised by osteoid formation, it is reasonable to find active BMP signalling in this tumour. Consistent with this finding, expression of BMP components was found in several benign and malignant bone tumours,[28,29] indicating that activation of the BMP pathway might not be related to malignant transformation. Here we confirm this by showing that pSmad 1 is expressed in the nuclei of most osteosarcoma cells indicating true activity of the pathway. Moreover by using luciferase reporters we demonstrate that the pathway is active and can be successfully modulated. This, however, does not affect the osteosarcoma cells' proliferation.

The role of TGFβ in MSC differentiation is not fully clear. It is generally thought that TGFβ increases bone formation by promoting the early stages of differentiation,[30] but inhibits mineralisation at later stage.[31] Studies in cancer have revealed a dual role of the TGFβ pathway as well, acting as a tumour suppressor in the initiation phase while promoting cancer progression in later phases.[10] Previously TGFβ was reported to stimulate osteosarcoma cell proliferation[32] and was related to aggressive behaviour and poor outcome in osteosarcoma patients.[33,34] However these studies only report on an association with the presence of ligands, which is not always indicative of functional pathway activity. Interestingly in this study stimulation or inhibition of the pathway did not affect osteosarcoma cells' proliferation. An explanation for this may be that in the previous report[32] cells were starved prior to stimulation by TGFβ rather indicating the normal reaction of starved cells to a growth factor than an osteosarcoma specific growth stimulation. In this study high pSmad2 expression in pre-treatment samples significantly correlated with better survival (Fig. 2). This might be related to recent discoveries showing immune-stimulating activity of the TGFβ pathway[35] which is in line with our recent results showing a significant role for the innate immune system in osteosarcoma's clinical course.[14,36]

Upregulation of Hh signalling has been implicated in cancer, especially in pancreas and basal cell carcinoma, but reports of Hh in sarcoma are scarce.[37] The only study investigating the role of Hh in osteosarcoma was performed on limited number of cases[38] and showed that Hh inhibition by cyclopamine inhibits osteosarcoma cells' proliferation. Accordingly *SMO* was indicated as a new therapeutical target for osteosarcoma. Our results show high variability of Hh activity measured by the expression levels of *PTCH1* and *GLI1* in cell lines (Fig. 3A) and clinical samples (Fig. 3C) which might be a result of the tissue heterogeneity found in osteosarcoma. Functional activity measured by a Hh reporter in osteosarcoma cell lines demonstrated high Hh activity only in OSA cell line, which could be attributed to an amplification at 12q, including the *GLI1*

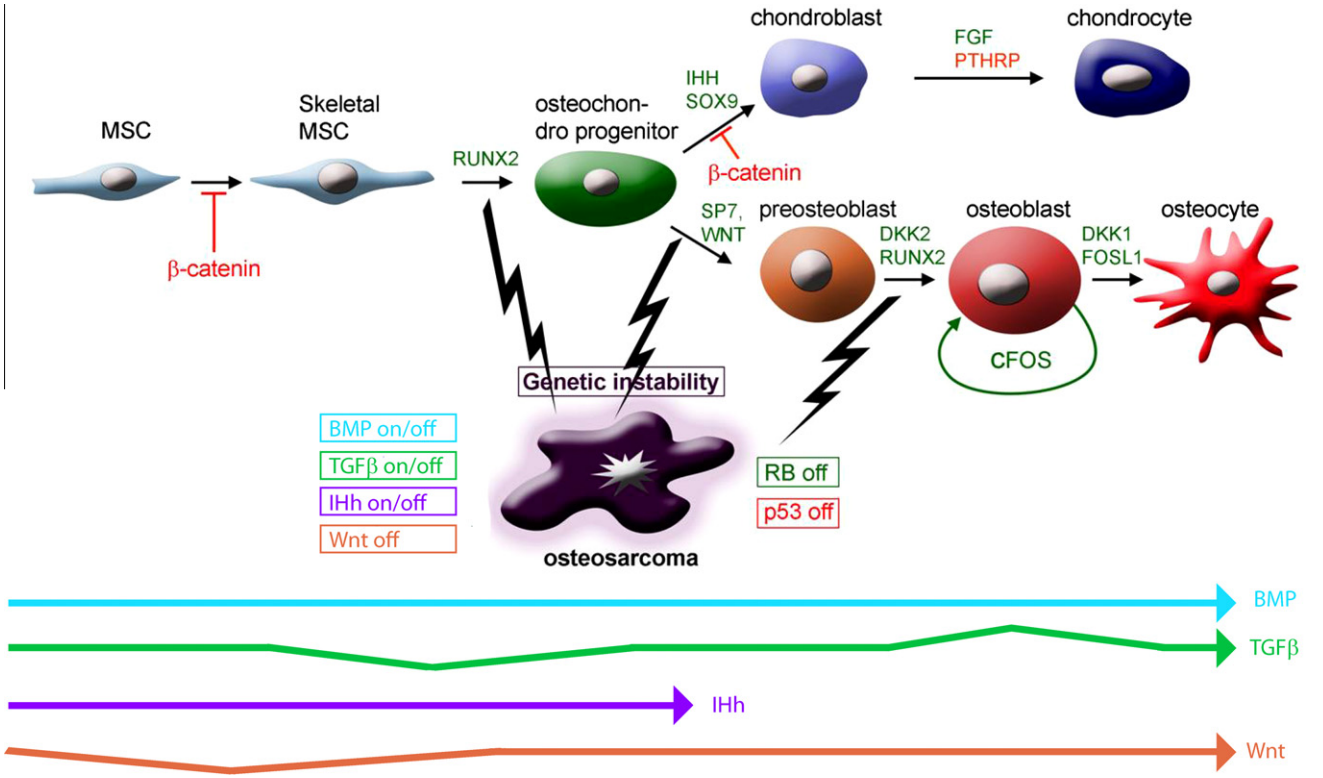

Fig. 7. Mesenchymal stem cell differentiation and osteosarcoma development. Schematic representation of several signal transduction pathways which play a role in skeletogenesis. The lines at the bottom depict the activity levels of these pathways during stem cell differentiation towards different lineages. Deregulation of one or more of these pathways may be involved in osteosarcoma-genesis.

gene.[39] However this amplification also includes *MDM2* and *CDK4* genes which are known, often amplified, oncogenes in osteosarcoma, indicating that this amplification may not be targeted at *GLI1*. The growth inhibiting effect of cyclopamine which we confirmed here, can be considered as an off-target, toxic effect for a number of reasons. Firstly, by knocking down the *GLI1* transcription factor and specifically decreasing Hh activity (shown by luciferase reporter and expression of target genes, Fig. 6) no inhibition of proliferation was detected. Secondly even though MG63 cells do not have any intrinsic Hh activity (Fig. 4), cyclopamine treatment resulted in decreased proliferation in these cells. Moreover after lowering the cyclopamine dosage, the proliferation inhibitory effect was lost in all 19 osteosarcoma cell lines (data not shown).

In conclusion, the current study clarifies the activity states of developmental pathways in osteosarcoma. We show that BMP/TGFβ signalling pathways are functionally active in the majority of osteosarcoma cases tested by the expression of phosphorylated Smads and in osteosarcoma cell lines tested by luciferase reporter systems. Interestingly a subgroup of osteosarcoma patients with low pSmad2 expression showed significantly worse disease specific survival, which might indicate a different role of the TGFβ pathway in regulation of anti-tumour immunity. Furthermore, our finding that cyclopamine inhibitory effect is independent of Hh activity explains the conflicting data in the literature on Hh targeted therapy in numerous cancers.

Role of the funding source

This work was financially supported by the Dutch Cancer Society (KWF) [Grant 2009-4012] and by EuroBoNet, a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumours [Grant LSHC-CT-2006-018814]. Authors declare that study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Authors' contributions

Study design: A.B.M., Y.C., P.tD., P.C.W.H. and A.C. Study conduct: A.B.M., Y.C., M.K., W.X., B.vdA., C.dA. and R.J. Data collection: A.B.M. Data analysis: A.B.M. and M.K. Data interpretation: A.B.M., P.C.W.H. and A.C. Drafting manuscript: A.B.M. Revising manuscript content: A.B.M., M.K., C.dA., P.tD., P.C.W.H. and A.C. Approving final version of the manuscript: All authors.

Conflict of interest statement

None declared.

Acknowledgements

The authors thank EuroBoNet partners for providing samples for gene expression analysis and Maayke van Ruler for technical support with the Smad stainings.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2012.06.018.

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