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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/127685 since 2020-04-15T16:49:08Z

Published version:

DOI:10.1002/jbmr.1578

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13 December 2025

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This is an author version of the contribution published on:

Dani N, Olivero M, Mareschi K, van Duist MM, Miretti S, Cuvertino S, Patane S, Calogero R, Ferracini R, Scotlandi K, Fagioli F, Di Renzo MF
The MET oncogene transforms human primary bone-derived cells into osteosarcomas by targeting committed osteo-progenitors
JOURNAL OF BONE AND MINERAL RESEARCH (2012) 27
DOI: 10.1002/jbmr.1578

The definitive version is available at:
http://doi.wiley.com/10.1002/jbmr.1578

The *MET* oncogene transforms human primary bone-derived cells into osteosarcomas by targeting committed osteo-progenitors

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Abstract: The *MET* oncogene is aberrantly overexpressed in human osteosarcomas. We have previously converted primary cultures of human bone-derived cells into osteosarcoma cells by overexpressing *MET*. To determine whether *MET* transforms mesenchymal stem cells or committed progenitor cells, here we characterize distinct MET overexpressing osteosarcoma (MET-OS) clones using genome-wide expression profiling, cytometric analysis, and functional assays. All the MET-OS clones consistently display mesenchymal and stemness markers, but not most of the mesenchymal–stem cell-specific markers. Conversely, the MET-OS clones express genes characteristic of early osteoblastic differentiation phases, but not those of late phases. Profiling of mesenchymal stem cells induced to differentiate along osteoblast, adipocyte, and chondrocyte lineages confirms that MET-OS cells are similar to cells at an initial phase of osteoblastic differentiation. Accordingly, MET-OS cells cannot differentiate into adipocytes or chondrocytes, but can partially differentiate into osteogenic-matrix-producing cells. Moreover, in vitro MET-OS cells form self-renewing spheres enriched in cells that can initiate tumors in vivo. MET kinase inhibition abrogates the self-renewal capacity of MET-OS cells and allows them to progress toward osteoblastic differentiation. These data show that *MET* initiates the transformation of a cell population that has features of osteo-progenitors and suggest that *MET* regulates self-renewal and lineage differentiation of osteosarcoma cells. © 2012 American Society for Bone and Mineral Research.

INTRODUCTION

The *MET* oncogene encodes the tyrosine kinase receptor of hepatocyte growth factor (HGF). The MET receptor and HGF are involved in tumor onset and progression, through their ability to orchestrate invasive cell growth under physiological and pathological conditions (for reviews, see 1, 2). *MET* has been shown to be activated by a point mutation in patients suffering from hereditary papillary renal cell carcinoma, a rare inherited cancer syndrome, and it is more commonly overexpressed in several tumor types (http://www.vai.org/met/). Moreover, we and others have shown that the *MET* oncogene is aberrantly overexpressed in a high percentage of human osteosarcomas, whereas it is almost not detectable in other mesenchyme-derived tumors, as well as in adult tissues of mesenchymal origin.3–7

Osteosarcomas are rare primary bone tumors, which occur primarily in children and adolescents (for review, see 8). Classically, they are defined by the production of an abnormal osteoid matrix, although most osteosarcomas are histopathologically graded as poorly differentiated. Osteosarcomas have chaotic karyotypes9 and lack both tumor-specific chromosomal translocations and definitive causative gene mutations, although mutations of *RB1* and *TP53* have been linked to osteosarcomagenesis in man (for reviews, see 10, 11) and mouse.12 Despite recent advances in osteosarcoma treatments, the patient prognosis for advanced disease remains poor.8

Several studies have provided evidence of the identification of osteosarcoma-initiating cells,13–16 the definition of which is crucial in the search for novel and more effective therapies and to characterize the genetic events that occur before the full-blown clinical manifestation. It is widely accepted that osteosarcomas derive from the mesenchymal stem cell lineage, as Ewing sarcomas do, although for the latter, the origin has been better defined. Ewing sarcomagenesis has been associated with the expression of the EWS–FLI-1 fusion protein in 85% of cases, which is all that is necessary for primary bone marrow-derived bona fide mesenchymal stem cells (MSCs) to be transformed and to become tumorigenic.17–20 In osteosarcomas, the presence of the osteoid matrix suggests that the target cells of osteosarcomagenesis might be committed progenitor cells. Indeed, it is still debated whether cancer stem cells derive only from the transformation of quiescent, long-term stem cells, or whether short-lived committed progenitor cells can aberrantly acquire the ability to undergo self-renewal.21, 22Studies of leukemogenesis support also the latter hypothesis,23 whereas the cell of origin of most solid tumors is still elusive.24, 25

We previously developed a model of osteosarcomagenesis in vitro,26 which we have used here to identify the target cells of *MET*transformation. We propagated bone-derived cells as primary cultures (HOB cells), which provided heterogeneous cell preparations where most of the cells were characterized by an osteoblast-like phenotype.27 Cells of these cultures are usually referred to as human osteoblast-like cells (HOBs). Using Lentivirus-driven gene transfer, we obtained overexpression of the *MET* oncogene in these cells, which converted them into osteosarcoma-like cells.26 Lentiviruses transduce bulk cell populations, give rise to random and different transgene insertions in each of the cells, and allow transgene expression driven by an internal promoter. *MET* overexpressing cells formed clones that carry multiple

copies of the *MET* transgene inserted in their genomic DNA. Southern blotting revealed random and distinct integration sites for*MET* transgenes, and demonstrated that each clone originated from distinct cells of the parental bone-derived cell population. All of these*MET* overexpressing clones had a transformed phenotype in vitro and showed the distinguishing features of human osteosarcomas in vivo, after their subcutaneous injection in immunodeficient mice. Therefore, we named these MET-OS clones.

Intriguingly, MET overexpression did not transform bona fide MSCs.26 Additionally, *MET*-induced transformation occurred in less than 0.1% of the transduced bone-derived cells, which suggested that there are specific and very rare susceptible target cells in the parental population. Here, we show the phenotypic characterization of the MET-OS clones using genome-wide expression profiling, cytometric analysis, and functional assays, and hence the identification of the cells that are targeted by *MET*-driven transformation. The advantage of this model is that from expression profiling we extract markers that are common to the few cells that are targeted by *MET*. Our data show that *MET* overexpression results in clonal expansion of a cell population that expresses markers of osteo-progenitors, and no longer markers of undifferentiated MSCs.

MATERIALS AND METHODS

Cell culture and reagents

The primary human HOB, MET-OS, and DN-MET-OS clones were obtained as described by Patanè and colleagues.26 All of the cell lines were routinely cultured in Iscove's Modified Dulbecco Medium (Sigma, St. Louis, MO, USA) supplemented with a 1% penicillin–streptomycin–amphotericin B mixture (Lonza, Versviers, Belgium), 1% L-glutamine (Lonza) and 10% fetal bovine serum (Sigma), at 37°C in a humidified 5% CO_2 atmosphere. JNJ-38877605 was obtained from Janssen Pharmaceutica/Ortho Biotech (Titusville, NJ, USA).

MSCs: preparation and differentiation

Bone marrow (BM) cells were harvested from the iliac crest of healthy donors and differentiated as described by Mareschi and colleagues.28 Whole BM was counted and plated directly in MSC Medium (Lonza) containing 10% fetal bovine serum (FBS) and seeded in T-25 flasks at the density of 100,000 cells/cm^2. After 5 days, non-adherent cells were removed and the attached cells had a medium change every 3 to 4 days. At each passage, the cells were counted and analyzed for cell growth and viability, and for their immunophenotype. The cells were labeled with the following antibodies: anti-human CD45, CD14, CD90, CD29, CD44, CD105, CD166, CD106, and CD73, and then washed with phosphate-buffered saline (PBS) before analysis with a FACScanto II (Becton Dickinson, San Jose, CA, USA), equipped with the

DIVA software program. The percentages of positive cells were calculated using the cells stained with IgG FITC/PE as a negative control.

For the differentiation experiments, the MSCs or MET-OS cells were cultured in osteogenic, adipogenic, chondrogenic, and neurogenic media (Lonza), according to the manufacturer instructions (Supplemental Fig. S2). Briefly, 200,000 and 300,000 cells were plated in T-25 flasks for osteogenic and adipogenic culture protocols, respectively. The cells were allowed to adhere for 24 hours in alpha-Minimum Essential Medium (α-MEM) plus 10% FBS, and then the medium was replaced with the complete specific induction medium. Osteogenic differentiation after 21 days was demonstrated by the accumulation of calcium (crystalline hydroxyapatite detection by Von Kossa staining). For the adipogenic differentiation, adipogenic induction and maintenance media were used alternatively every 3 to 4 days. The presence of intracellular lipid vesicles was assessed by oil red O staining. For chondrogenic differentiation, 250,000 cells were washed twice in incomplete chondrogenic medium in polypropylene culture tubes, and then resuspended in complete chondrogenic medium in the presence of transforming growth factor (TGF)-β3. Chondrogenic differentiation was demonstrated by cell growth as aggregates, which floated freely in suspension culture. The pellet was included in paraffin and stained with Alcian blue to identify hyaluronic acid and sialomucin. To differentiate the MSCs towards a neural lineage, they were treated as described by Mareschi and colleagues.29Semiconfluent adherent cells were detached and washed with PBS. Then 250,000 cells were plated in T-25 flasks in the presence of Neural Progenitor Maintenance Medium. This medium was supplemented with recombinant human basic fibroblastic growth factor (hFGF-b), recombinant human epidermal growth factor (hEGF), neural survival factor-1 (NSF-1), 30 mg/mL gentamicin, and 15 μg/mL amphotericin. Under these conditions, the MSCs acquire new morphological characteristics, neural markers, and electrophysiological properties, which indicate their neural differentiation.29

Quantitative determination of calcium accumulation

To evaluate calcium deposition, the matrix was demineralized by addition of 0.6 N HCl during an overnight incubation at 37°C. The solutions were then collected and centrifuged at 2,000 × *g* for 5 min. The calcium concentrations in the supernatant were determined by colorimetry using a complexometric method based on o-*cresol-ftalein*. After decalcification, the cells were washed three times in PBS and then solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulphate. The protein content was measured with BCA Protein Assay kits (Pierce, Rockford, IL, USA). The calcium content of the cell layer was normalized to total cellular protein.

RNA preparation and quantitative reverse transcription (qRT) polymerase chain reaction (PCR)

Total RNA was extracted and purified from exponentially growing cells using RNeasy Mini-kits (Qiagen, Hilden, Germany) and quantified and inspected using Bioanalyzer analysis (Agilent Technologies, Waldbrom, Germany). The cDNA for quantitative reverse transcription-PCR was synthesized from the total RNA using SuperScript VILO cDNA Synthesis kits (Invitrogen, Carlabad, CA, USA), according to the manufacturer protocols. The qRT-PCR reactions were carried out in triplicate on a MyIQ single color real-time PCR detection system (BioRad, Hercules, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). For each sample, 1 µL cDNA (corresponding to 500 ng RNA) was incubated in a final volume of 25 µL with 1× SYBR Green PCR Master Mix and both forward and reverse target-specific primers (Supplemental Table S3). The thermal cycling conditions used to validate gene expression changes were: hold for 10 minutes at 95°C, followed by three-step PCR for 40 cycles of 95°C for 15 seconds, 55°C to 60°C for 25 seconds, and 72°C for 30 seconds. The expression of each target gene was evaluated using a relative quantification approach (−DDCt method; Livak, 2001) with β-actin as the internal reference.

Microarray sample preparation

cRNAs for microarray were generated and hybridized on Illumina DNA chips, according to the manufacturer's instructions. The chips were scanned with a specific scanner (Illumina BeadArray Reader, Illumina Inc., San Diego, CA, USA) to generate digitized image data files. The expression profiles of the samples of interest were analyzed using the BeadStudio software package included with the Illumina Gene Expression System. BeadChip array data quality control was performed using Illumina BeadStudio software, version 1.3.1.5. The transcript average intensities were calculated using Illumina BeadStudio software, and were normalized by the Rank-Invariant Method, and $\log_2$transformed. Principal component analysis and hierarchical clustering (ST, Euclidean distance, average clustering, 5000 jackknife resampling steps) were performed using the Multi Experiment Viewer 4.1 application (http://www.tigr.org/software/). Differentially expressed genes were identified using the SAM methods implemented in the above program. The validation of microarray data was performed with qPCR as described above, using primers for the selected genes reported in Supplemental Table S3.

Sphere formation assay

The spherical colony formation assays were performed as described by Gibbs et al.,30 with some modifications. Briefly, the cells were plated at 800 cells/cm^2 into ultralow attachment plates (Corning Inc., Corning, NY, USA) in serum-free DMEM/F12 medium (Invitrogen), supplemented with 1 mM penicillin and 100 U/mL streptomycin, 2 mM L-glutamine, 10 ng/mL hFGF-b, 20 ng/mL EGF, 4 mg/mL bovine serum albumin, 4 µg/mL heparin, 1% B27, and 0.5%

N2 supplements (Invitrogen). The cells were cultured at 37°C in 5% CO_2 without replacing the culture medium. Fresh aliquots of EGF and hFGF-b were added every 2 days. After culturing for 48 to 72 hours, the spheres start to be visible under an inverted phase-contrast microscope. On day 14 the spheres were counted. Counting was performed in a blinded manner, and three-independent experiments were performed.

Tumorigenicity assay

In vivo studies were conducted in 5- to 7-week-old severe combined immunodeficiency (NOD-SCID) and NOD-SCID IL2rg−/− female mice (Charles River Italia, Como, Italy). The indicated numbers of adherent cells or floating sphere-derived cells were suspended in a solution of culture medium and Matrigel (1:1; Becton Dickinson) and injected subcutaneously into the right flank of mice, under anesthesia. Tumor growth was assessed twice weekly, and the mice were sacrificed 2 to 6 months after the injections. Before xenotransplantation, the viability of the samples was verified by flow cytometry, after propidium iodide incorporation. All animal procedures had been approved by the Ethical Commission of the University of Torino and by the Italian Ministry of Health.

Statistical analysis

The results are presented as means ± SD. Statistical differences were determined using student's *t*-tests or one-way analysis of variance (ANOVA). Probabilities of less than 5% ($p < 0.05$) were considered significant.

RESULTS

The MET-OS clones share features with a group of human osteosarcoma cell lines

We and other have shown that the *MET* oncogene is aberrantly overexpressed in approximately 80% of all subtypes of human osteosarcomas,3–7, 31 although it is almost not detectable in other mesenchyme-derived tumors, such as fibrosarcoma and chondrosarcoma, as well as in adult tissues of mesenchymal origin.3–7 To study MET-induced transformation of cells derived from mesenchymal lineages, we overexpressed the MET oncogene in primary cultures of human bone-derived cells and obtained MET overexpressing osteosarcoma (MET-OS) clones.26 As previously reported, the MET-OS clones originated from independent and multiple events of *MET* transgene integration into cultured cells.26 This implies that these clones were derived from distinct cells in the heterogeneous parental populations, which then underwent propagation and in vitro transformation.

To characterize the phenotype of the MET-OS clones, we used an oligonucleotide microarray approach. The expression profiles of a number of the MET-OS clones and several human osteosarcoma cell lines were compared in this way. The osteosarcoma cell lines studied had been previously and extensively characterized, in particular, by the EuroBoNeT consortium32–34 and found to be representative for clinical osteosarcoma. Indeed, these cell lines have been characterized as far as molecular profiles, differentiation properties in vitro and tumorigenicity in vivo. We and others demonstrated also that all these cell lines express the MET receptor at high level, although it is poorly expressed in mesenchymal stem cells and differentiated osteoblasts.3, 26 As shown in Figure 1*A*, the MET-OS clones (indicated according to their original clone numbers; for example, C6, C8, etc) clustered together with some of the osteosarcoma cells lines, such as the Saos-2 cells, while other cell lines formed a distinct cluster, which included the U-2 OS cells. These data indicated that the MET-OS clones are phenotypically analogous to a group of osteosarcoma cell lines, and that they might thus be suitable to study the role of the *MET* oncogene in the development of osteosarcoma.

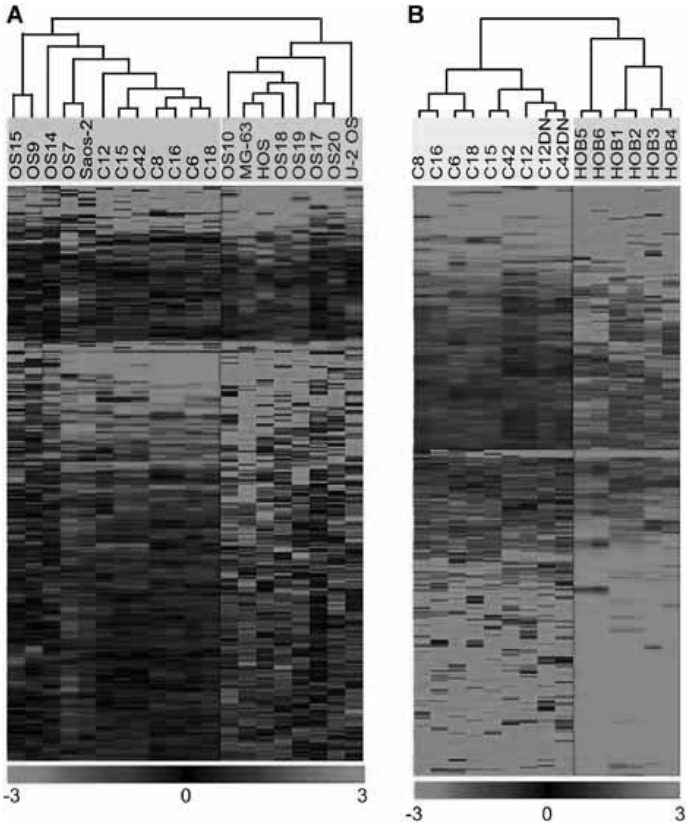


Figure 1 Microarray data-clustering of the human MET-OS clones, compared with the osteosarcoma cell lines (*A*) and to the parental bone-derived cells (*B*). Bioinformatic analysis of the indicated cell samples was performed using the Multi-Experiment Viewer 4.1 application. The cluster analysis organized the samples according to the similarities and dissimilarities of their expression profiles, placing the samples with similar expression profiles together as neighboring columns in the clustergram (at*top*). (*A*) Comparison of the MET-OS clones and the osteosarcoma cell lines (listed at*top*). The hierarchical clustering of 300 genes differentially expressed >3 $\log_2$-fold shows grouping of the MET-OS clones (C6, C8, C12, C15, C16, C18, C42) with a group of osteosarcoma cell lines (Saos-2, OS7, OS15, OS9, and OS14, green). These sample groups are both clearly distinct from the second group of osteosarcoma cell lines (red). (*B*) Comparison of the MET-OS clones and the parental bone-derived cells (HOB 1–6, indicated according to their original preparation numbers). The hierarchical clustering of the 311 genes differentially expressed >3 $\log_2$-fold shows that the MET-OS clones (blue) are clearly separated from the HOBs (pink). The DN-MET-OS clone variants (C12DN, C42DN) clustered with the MET-OS clones from which they were derived (GSE28256; GSE28252; http://www.ncbi.nlm.nih.gov/geo/).

The MET-OS clones all derive from distinct progenitor cells with a stem phenotype

To characterize the cells from which the MET-OS clones originated, their expression profiles were compared with those of the primary cultures of the parental bone-derived cells from which they were derived, that is, the above-mentioned HOBs. As shown in Figure 1*B* and Supplemental Figure S1, the MET-OS clones showed homogeneous patterns of expression, although they were derived from single and distinct cells. Moreover, their expression profiles differed greatly from those of the HOBs. Furthermore, the parental HOBs clustered together, even if they are expected to be relatively heterogeneous and to contain MSCs, osteoblast-lineage-committed cells, and some other lineage-committed cells derived from MSCs.27, 35 Indeed, although in all parental HOB cultures more than 80% of cells showed alkaline phosphatase expression,26 we found that these cultures contained 2% to 10% bona fide MSCs, which express markers such as CD105, CD73, CD44, and CD166 and a high expression of CD29 (data not shown).

Analysis of the expression-profile data shows that 534 genes were differentially expressed >2 $\log_2$-fold in the MET-OS clones versus the HOBs (Supplemental Table S1), with 311 genes differentially expressed >3 $\log_2$-fold (Fig. 1*B*). To rule out the possibility that these differences between the MET-OS clones and the HOBs were because of transformation or simply to *MET* overexpression, the expression profiles were also analyzed for the MET-OS clones when their MET kinase activity was quenched with a dominant-negative *MET* approach (DN-MET-OS clones).26 We have previously demonstrated that this DN-MET expression abolishes the MET-dependent biological properties of the MET-OS clones and impairs their in vivo tumorigenicity.26 As shown in Figure 1*B*, the expression profiles of the MET-OS clones and the corresponding DN-MET-OS clones still clustered together (Fig. 1*B*; C12DN and C42DN). This demonstrates that the expression profiles of the MET-OS clones are indicative of the phenotypes of the individual cells that were targeted by the *MET*-driven transformation, rather than being associated with either *MET* overexpression or transformation.

To identify the *MET*-targeted cell population, we used these microarray data and qPCR and FACS analyses. We examined the expression of specific groups of genes that have been associated with the mesenchymal and stemness phenotype of MSCs, and with the osteo-progenitor and/or osteoblast phenotypes.

Table 1 shows that in all the MET-OS clones analyzed with microarrays, the markers associated with the MSC phenotype (*CD29, CD44, CD73, CD90, CD105, CD106, CD166*) were less expressed than in the parental HOB preparations. Quantitative PCR and FACS analyses confirmed these results. Figure 2 shows representative FACS plots of the MET-OS clone C42. In contrast, *CD117*, a marker of the mesenchymal lineage that has recently been associated with osteosarcoma cell stemness,15 was more expressed in the MET-OS clones, with respect to HOB preparations (Table 1). Two other markers of stemness, *POU5F1* (also known as *OCT3/4*) and *ABCB1* (also known as*MDR1*), were more expressed and de novo expressed, respectively, in the MET-OS clones. *CD133* was not expressed (data not shown), whereas the *RB1* transcript, the loss of which has been associated with MSC differentiation along lineages other than osteoblastic,36 was expressed in HOBs at low levels, which were

nevertheless detectable, but not found in the MET-OS clones, as in most human osteosarcomas.10, 11 Markers of hematopoietic stem cells (*CD34, CD45*) were not expressed in either the MET-OS clones or HOBs (data not shown).

Gene symbol	Gene description	Fold change
Group a:		
CD29	integrin, beta 1 (ITGB1)	0.294
CD44	cell surface glycoprotein CD44, transcript variant 1	0.158
CD73	5'-nucleotidase, ecto (NT5E)	0.049
CD90	Thy-1 cell surface antigen (THY1)	0.004
CD105	endoglin (ENG)	0.027
CD106	vascular cell adhesion molecule 1 (VCAM1)	0.009
CD166	activated leukocyte cell adhesion molecule (ALCAM)	0.147
CD117	v-kit feline sarcoma viral oncogene homolog (KIT)	17.128
RB1	retinoblastoma 1	not detectable in MET-OS
ABCB1	ATP-binding cassette, subfamily B (MDR/TAP)	neo-expressed[a]
POU5F1	POU class 5 homeobox 1, isoform 1 (OCT3/4)	2.722[a]
Group b:		
CDKN2A	cyclin-dependent kinase inhibitor 2A	14.074
CDKN3	cyclin-dependent kinase inhibitor 3	8.095
CDCA4	cell division cycle associated 4	7.594
Group c:		
RUNX2	runt-related transcription factor 2	5.244
ALPL	alkaline phosphatase, liver/bone/kidney	7.841
MATN2	matrilin 2	10.348
FN1	fibronectin 1	0.044
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	0.071
COL1A1	collagen, type I, alpha 1	0.111
COL1A2	collagen, type I, alpha 2	0.024
Group d:		
SPP1	secreted phosphoprotein 1 (osteopontin)	low and not changed[a]
BGLAP	bone gamma-carboxyglutamate protein (osteocalcin)	low and not changed[a]

Table 1 Differentially Expressed Genes in the MET-OS Clones Versus HOB
Differential expression of markers of MSC stemness (Group a) and of different stages of osteoblastic differentiation, selected on the basis of the literature. Group b: markers of the early/proliferative phase, Group c: markers of the intermediate phase; Group d; markers of the late phases.
A As determined by qPCR.

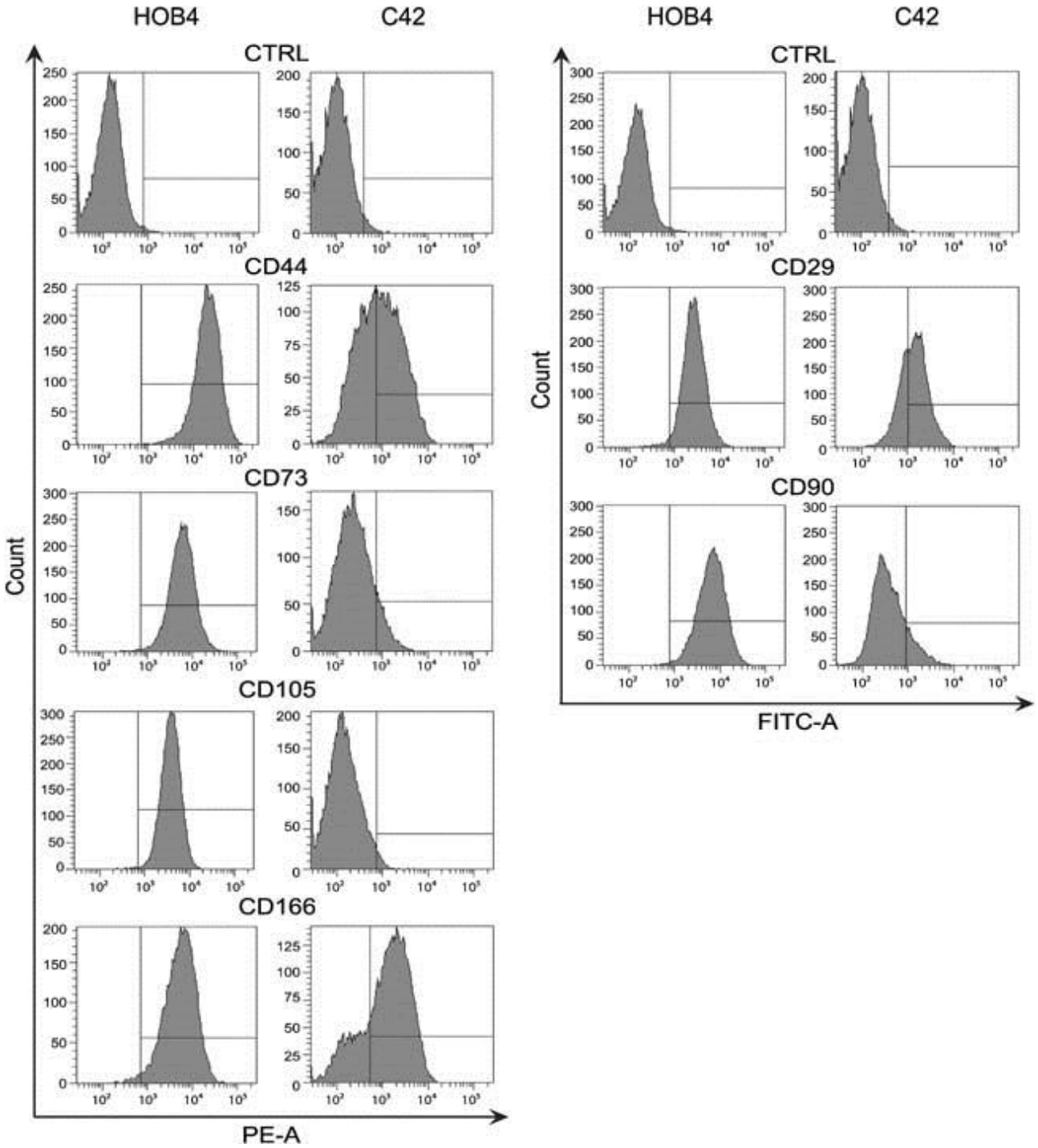


Figure 2 Differential expression of phenotypic MSC markers in the MET-OS clones versus the parental bone-derived cells. FACS analysis shows the different expressions of the MSC markers (as indicated) in the MET-OS clone (C42) and in the parental bone-derived (HOB4) cells.

Table 1 also shows that genes related to the early proliferative phase of osteoblastic differentiation (*CDKN2A, CDKN3, CDCA4*37), and some genes related to the intermediate differentiation phase (*RUNX2, ALPL, MATN2*), were more expressed in the MET-OS clones than in HOB preparations. In contrast, other genes of the intermediate differentiation phase (*FN1, SPARC, COL1A1, COL1A2*) were less expressed in the MET-OS clones, whereas genes associated with the late phase of matrix maturation and mineralization (*SSP1, BGLAP*) were barely detectable in both the MET-OS clones and HOBs.

Altogether, these data suggested that the MET-OS clones originated from the selective expansion of a distinct subpopulation of cells with the phenotype of osteo-progenitors. Moreover, MET-OS clone expression profiling demonstrated that MSCs are not likely to be the target cells of the *MET*-driven transformation.

The MET-OS clones are derived from a committed osteo-progenitor at an early stage of differentiation

The osteogenic differentiation process consists of a well-coordinated multistep sequence of events in which each stage is characterized by expression of distinct protein markers and by individual morphological features.38 To identify the step at which cells of the mesenchymal lineage are permissive to the *MET*-driven transformation, we used expression profiling again. Here, we compared the transcription profiles of the MET-OS clones and MSCs, and MSCs induced toward osteoblastic differentiation (Fig. 3). The stemness of the parental MSC preparations was shown by inducing differentiation of the same cell preparations in parallel, towards osteocyte, adipocyte, chondrocyte, and neuron-like lineages (Fig. 3, Supplemental Fig. S2). Osteoblastic differentiation was obtained by culturing MSCs in the presence of osteogenic factors (ascorbic acid, β-glycerophosphate and dexamethasone) for up to 21 days.39 The MSC cultures induced to differentiate in this way were collected after 7, 14, and 21 days. Differentiation of the MSCs was inspected through the whole period, under light microscopy and osteogenic differentiation was confirmed by the presence of signs of calcification, which first appeared after 7 days, as black regions within the cell monolayer. Calcified extracellular matrix (the osteomatrix) was abundant after 21 days of treatment (Fig. 3*A*; Von Kossa staining).

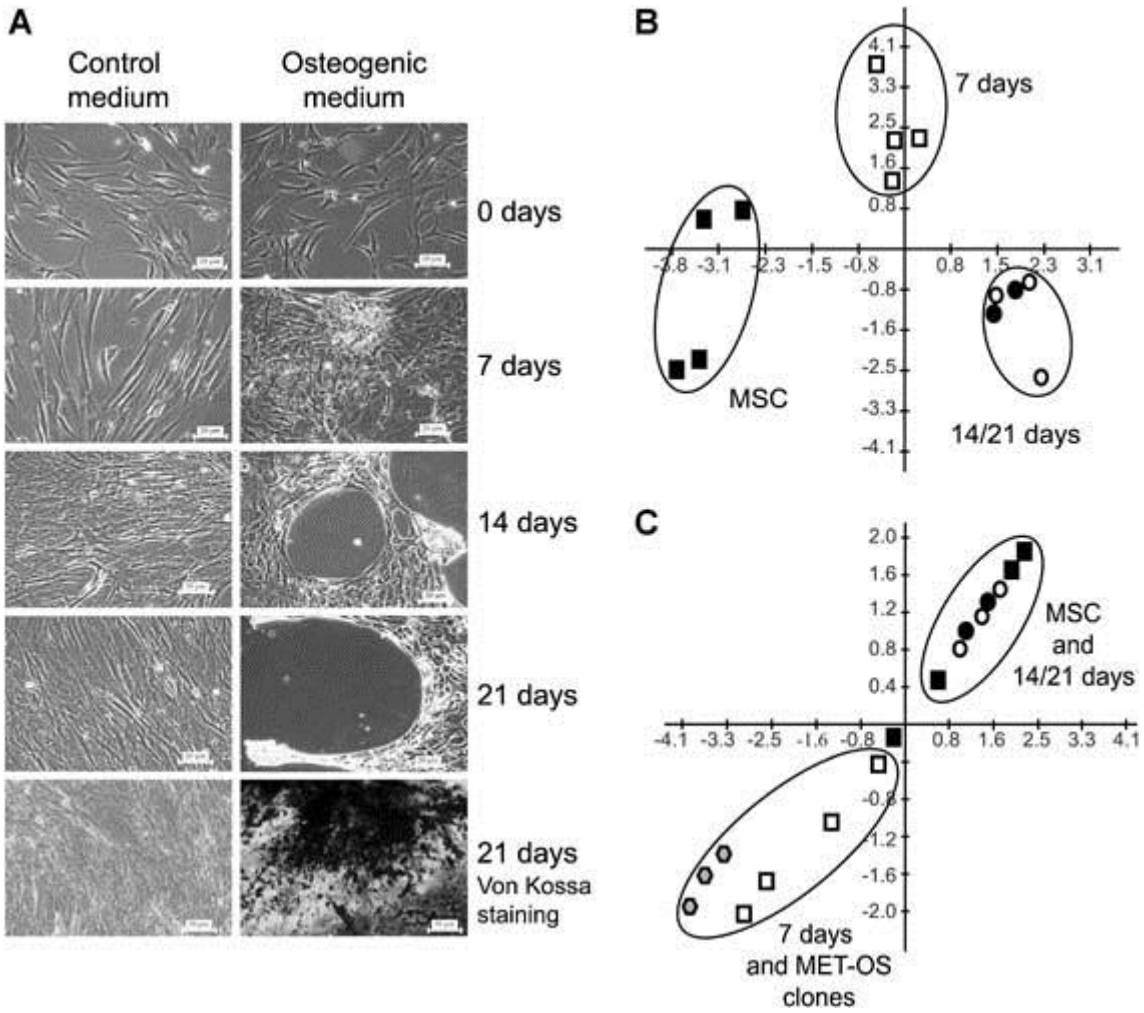


Figure 3 Hierarchical clustering of the osteoblastic differentiation stages. (A) MSCs were cultured in the absence (control medium) or presence of osteogenic differentiation medium, containing 50 µg/mL ascorbic acid, 3.5 mM β-glycerophosphate and 10 nM dexamethasone, for 7, 14, and 21 days. The production of the osteomatrix was detectable after 14 and 21 days, as further demonstrated by Von Kossa staining. (B) Principal component (PC) analysis of the full expression profiling of the differentated samples shown in the panel A. One projection of the PC analysis of the whole microarray data set is shown. Cluster analysis organizes the cell samples according to the similarities or dissimilarities of their expression profiles, placing the samples with similar expression profiles together in this PC analysis. The values on the X and Y axes are ×102. The hierarchical clustering reveals three main phases of osteogenic development: undifferentiated MSCs (closed squares), 7-day differentiated cells (open squares), and 14/21-day differentiated cells (open/closed circles). (C) Principal component analysis of differentiated samples and the MET-OS clones (C12, C15, and C42) using the 7-day differentiation-specific genes (gene set B in Supplemental Table S2). The hierarchical clustering shows a clustering of clones (gray squares) together with the 7-day differentiated cells (open squares) (GSE28205; http://www.ncbi.nlm.nih.gov/geo/).

The expression profiles of MSCs and of MSC cultures undergoing induction of osteoblastic differentiation allowed their separation into three clusters that were representative of three differentiation phases. These three clusters were: the undifferentiated MSCs, and the 7-day

differentiated and 14/21-day differentiated cells that were derived from MSCs (Fig. 3*B*). This indicated that at the transcriptional level, after 14 days, the differentiated cells were nearly identical to those differentiated for 21 days. Therefore, in the further analyses, the 14-day and 21-day samples were considered together. Supplemental Table S2 shows that 182 genes distinguished the MSCs from the 7-day-induced plus 14/21-day-induced cells (gene set A of Supplemental Table S2), 96 genes distinguished the 7-day-induced cells from the MSCs and the 14/21-day-induced cells (gene set B), and 53 genes distinguished the 14/21-day-induced cells from the MSCs and the 7-day-induced cells (gene set C). The MET-OS clones clustered together with the 7-day-induced cells when gene set B was analyzed (Fig. 3*C*). When gene sets A or C were analyzed, the MET-OS clones did not cluster with the MSCs or with any of the groups of induced cells (data not shown). These data were also validated by qPCR, using primers for a randomly selected set of 10 of the 96 genes of set B (data not shown). These data show that the MET-OS clones are similar to cells at an initial phase of osteoblastic differentiation.

As mentioned above, under the appropriate in vitro conditions, MSCs can also differentiate into adipocytes and chondrocytes.29, 40, 41 We thus used qPCR to determine whether the MET-OS clones and MSCs induced to differentiate into osteoblasts share markers with other differentiation pathways after 7 days of induction of differentiation towards adipocytes and chondrocytes. The MET-OS clones shared expression of a number of transcripts with all of the differentiation lineages, such as the stemness markers *DLG7*, *MELK,* and *PBK* (Fig. 4*A*), whereas markers of lineage specificity were differentially expressed. As shown in Figure 4*B*, the MET-OS clones only expressed differentiation markers associated with an osteoblast lineage, such as *RUNX2* and *SP7* (also known as Osterix), and not adipocyte markers (*LPL, PPARG2*) or the chondrocyte marker *CCN5.* Interestingly, *SOX9*, which is considered to be the most indicative chondrocyte marker, was expressed by the MET-OS clones, although at low levels (Fig. 4*B*). This is in agreement with its reported early expression during MSC differentiation into all lineages, before the full activation of *RUNX2* during osteoblast differentiation. In agreement, expression of MATN2, which is regulated by SOX9, is increased in MET-OS, whereas collagens and other ECM members are only moderately expressed in MET-OS. In addition, we found that the *RUNX2* expression level in the MET-OS clones was comparable to that of osteosarcoma cell lines, such as the Saos-2, OS9, OS15, and OS17 cell lines, and higher than that of the MG63 osteosarcoma cell line (data not shown), in agreement with data reported in other studies.42 These data demonstrate that the cells that are prone to *MET*-driven transformation are early, but committed, osteo-progenitors.

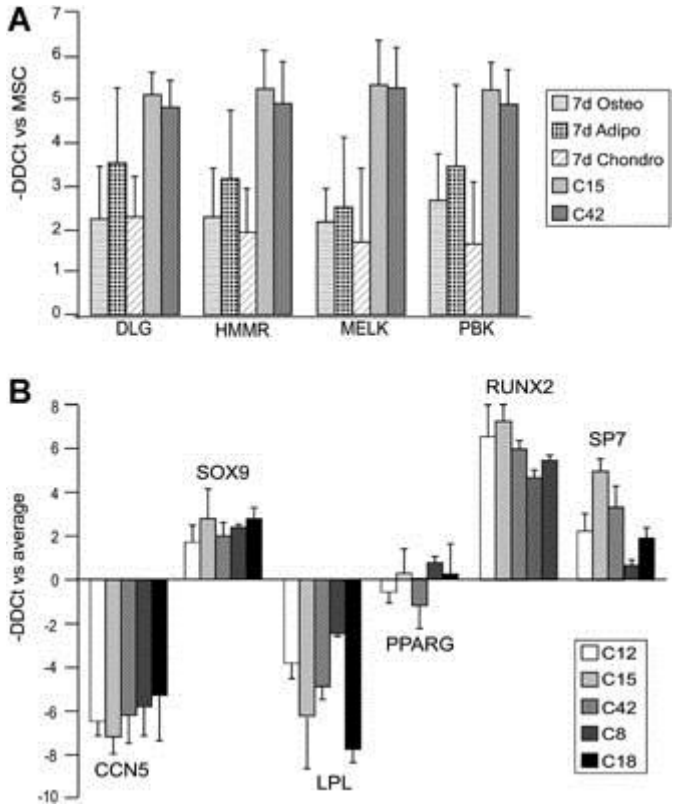
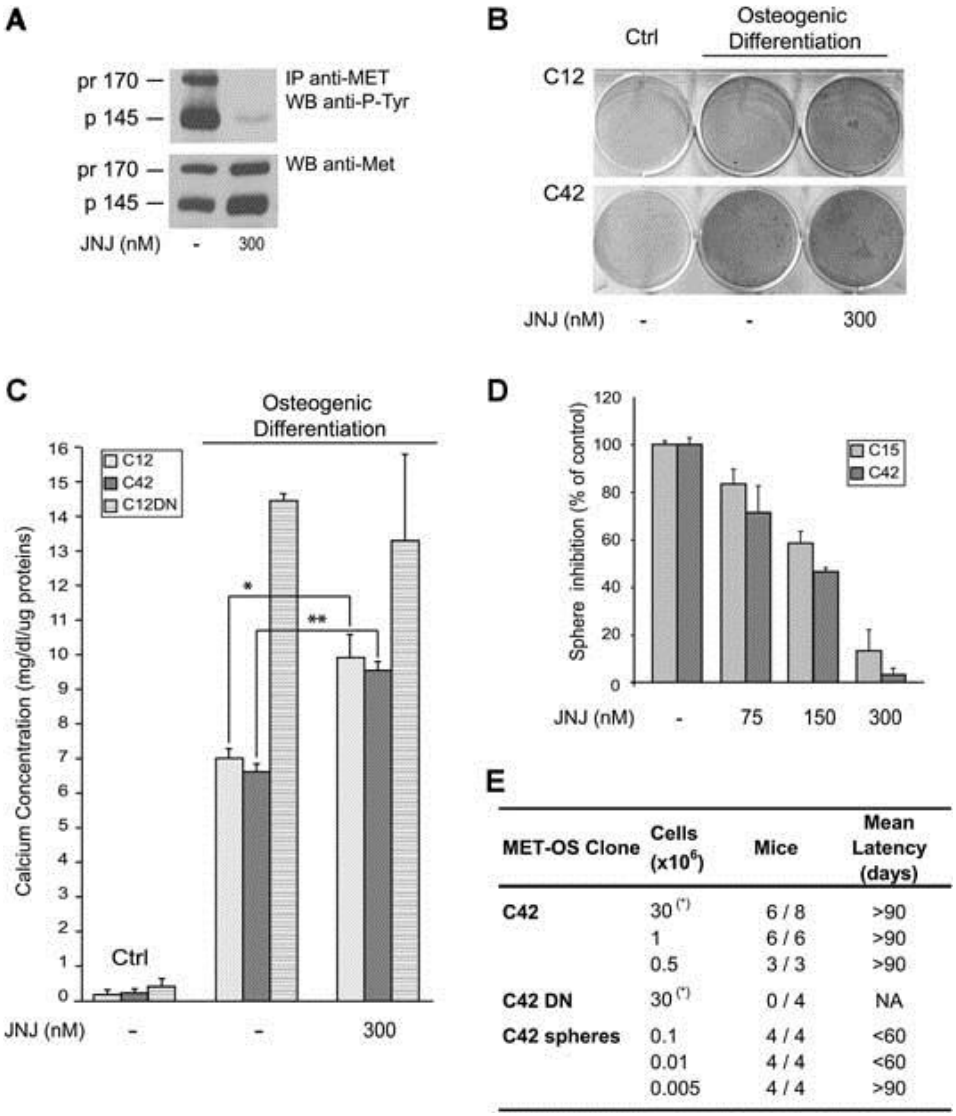


Figure 4 Expression of differentiation markers in the MET-OS clones measured using qPCR. (A) A number of markers associated with stemness on the basis of literature data were similarly expressed in all of the differentiation lineages after 7 days of MSC exposure to differentiating media, and overexpressed in the MET-OS clones C15 and C42. (B) Conversely, the markers associated with lineage specificity, which are expressed in each lineage after more prolonged cell exposure to the differentiating media, are specifically either overexpressed or absent in the MET-OS clones.

As the MET-OS clones expressed markers in common with early chondro-progenitor cells and adipo-progenitor cells, we tested whether the MET-OS cells maintain the ability to differentiate along the MSC-derived lineages. Growing the MET-OS clones in lineage-specific differentiating media resulted in a lack of differentiation along the chondrocyte and adipocyte lineages, but gave rise to production of some osteogenic matrix (Fig. 5*B*, middle wells).



MET-OS Clone	Cells ($x10^6$)	Mice	Mean Latency (days)
C42	30 (*)	6 / 8	>90
	1	6 / 6	>90
	0.5	3 / 3	>90
C42 DN	30 (*)	0 / 4	NA
C42 spheres	0.1	4 / 4	<60
	0.01	4 / 4	<60
	0.005	4 / 4	>90

Figure 5 The self-renewal ability and differentiation potential of the MET-OS clones depend on MET activation. (A) The MET kinase small-molecule inhibitor JNJ-38877605 abrogated MET activation in the MET-OS clone C42, where MET was constitutively activated because of its overexpression.26 JNJ-38877605 (B–C) and the expression of a dominant-negative form of the MET receptor (C12DN) (C) allowed cell progression towards osteoblastic differentiation, as demonstrated by osteomatrix production (B) and quantified as calcium released by the cells (C), after 14 days of osteogenic induction. (D) JNJ-38877605 inhibited sphere formation in the MET-OS clones (C15 and C42) seeded at low density under serum-free conditions. (E) Injection of the MET-OS clones into immune-compromised mice resulted in xenograft growth: NOD/SCID (C42 and C42 DN) and NOD-SCID IL2rg−/− (C42 spheres) have been injected. The number of mice with tumors out of the mice injected is shown. (*) data already shown in 26.

Altogether, these data show that the cells targeted by *MET* transformation are osteo-progenitor cells that lack multilineage potential, but that retain the ability to undergo osteoblastic differentiation.

MET overexpression inhibits full osteoblast differentiation and sustains self-renewal of the MET-OS cells

Conflicting reports have described the HGF/MET receptor pair as either inhibiting43 or inducing44, 45 osteoblast differentiation. Therefore, we tested the ability of cells of the MET-OS clones, which are derived from osteo-progenitor cells, to undergo differentiation into mature osteocytes in the presence of the highly specific, small-molecule MET kinase inhibitor, JNJ-38877605.46, 47 This was investigated using Von Kossa staining and the measurement of calcium levels.

The cells were grown in the appropriate differentiating media in the presence or absence of 150 nM or 300 nM JNJ-38877605. MET inhibition (Fig. 5*A*) did not affect the survival of the MET-OS cells (not shown), but it resulted in their producing increased amounts of calcified matrix (Fig. 5*B*).

We have already shown that *MET* overexpression in the MET-OS clones is required for cell anchorage-independent growth in a semisolid medium in the presence of serum.26 Here, we evaluated the potential of the MET-OS cells to form non-adherent spheres in serum-free medium containing only EGF and FGF-b, a "sphere-forming" assay that evaluates the cells that can undergo self-renewal. The MET-OS cells were plated at a clonogenic density in non-adhesive flasks, under conditions optimized to preclude reaggregation of single cells. Starting from 7 days of culture, all of the MET-OS cells formed spheres, which could be serially passaged up to five times. In the presence of 75 nM to 300 nM of the MET inhibitor JNJ-38877605, the formation of these spheres by the MET-OS cells was almost abolished (Fig. 5*D*). Osteosarcoma cell lines similarly formed spheres in the same conditions, although at a different extents. This sphere forming ability was similarly inhibited by JNJ-38877605 (Supplemental Fig. S3*A*).

We then examined whether the MET-OS cells can initiate tumor formation when grafted at decreasing densities in immune-compromised mice. It is well known that human osteosarcomas and osteosarcoma cell lines grow at very low efficiency even in nude mice. Thus, high numbers of cells are necessary to obtain xenografts. Indeed, lack of the correct microenvironment when these cells are injected subcutaneously, and the innate immunity, are probably the major limiting factors in assays of the tumorigenicity of osteosarcoma cells in vivo. Therefore, we used improved xenograft assay conditions that have been developed to measure tumorigenicity of melanoma cells,48that is, more severely immunocompromised mice (either NOD-SCID or NOD-SCID IL2rg−/− mice) and cell transplants in the presence of Matrigel (an artificial basement membrane of collagen, laminin, and glycosaminoglycans). Under these conditions, subcutaneous tumor formation was induced at the lowest cell numbers tested (Fig. 5*E*). Furthermore, as the non-adherent spheres of the MET-OS clones are thought to be enriched with cells with self-renewal ability, we also tested their tumor-forming abilities. Figure 5*E* shows that tumors grew also when 5×10^3 MET-OS cells derived from these spheres were injected subcutaneously. Altogether these data support the hypothesis that the MET-OS clones contain cells with self-renewal ability. By contrast, the expression of DN-MET abolished tumourigenicity of the MET-OS clones even at the highest cell

concentrations (30×10^6 cells; Fig. 5*E*),26 and this also suggest that self-renewal ability of the MET-OS clones relies on *MET* activation. Accordingly, the tumorigenicity of osteosarcoma cell lines overexpressing MET was abolished by the expression of DN-MET (Supplemental Fig. S3*B*).

DISCUSSION

To identify the cells targeted by *MET* transformation, we started with expression microarrays, and compared the MET-OS clones to the parental HOBs from which they were derived. There was notable intra-group identity and remarkable inter-group differences. In contrast, the microarray-based expression profiles of the MET-OS clones were almost identical to those of their DN counterparts, the DN-MET-OS clones. These DN-MET-OS clones consisted of an unselected bulk-cell population that was obtained from the MET-OS clones after transduction with a Lentiviral vector carrying the DN-*MET* transgene. This block of MET activation effectively turned off both the transformed and the tumorigenic phenotypes of the MET-OS clones.26 Therefore, our data suggest that the expression profiling reflects the phenotype of the individual cells of origin of the MET-OS clones and does not depend on *MET* expression, MET activation or MET-driven transformation. The intra-group identity among several of the MET-OS clones was indicative of the origin of all of these clones from a single cell type, although they were derived from distinct cells. This further supports the origin of the MET-OS clones from a specific sub-population, and it suggests that *MET* expression favored the selective transformation of this sub-population.

Expression profiling of the MET-OS clones, MSCs and MSCs induced to differentiate along different lineages allowed the identification of a number of markers of the sub-population prone to *MET* transformation. Markers of the MSC phenotype, such as *CD29*, *CD44*, and *CD90*, were all down-modulated in the MET-OS clones, with the notable exception of *CD117*, which has been already identified as a marker of mesenchymal cells and associated to osteosarcoma cell stemness.15 In addition, the MET-OS clones did not express the stem-cell marker*CD133*, which characterizes Ewing sarcoma initiating cells, believed to be of MSC origin.17–20 *CD133* was also found expressed in a few human osteosarcoma-derived cell lines,16, 49 suggesting that a sub-group of human osteosarcomas actually derive from bona fide MSCs. Finally, cells of the MET-OS clones have lost their multilineage potential. We concluded that undifferentiated MSCs are not likely the primary target of the *MET*-driven transformation. In agreement with this, there was no transformation of MSCs upon *MET* overexpression.26

We thus focused on genes that are associated with the osteoblast lineage. Osteoblast differentiation consists of a number of phases that are characterized by sequential expression of lineage-specific transcription factors. These drive the expression of proteins, such as collagen, osteopontin, and osteonectin, which are characteristic of the differentiated cells. The MET-OS clones showed expression profiles and markers of the 7-day-induced osteoblastic

lineages of MSCs. Among these markers, *LPL* and *PPARG* were suppressed, but *SOX9* was shown to still be expressed when *RUNX2* expression was increasing. These expression patterns are highly similar to those reported to be characteristic of the early phases of osteogenic induction.50, 51 In addition, the MET-OS clones have been shown to have high expression of alkaline phosphatase,26 an early and non-specific marker of osteoblast lineage. In contrast, late markers of osteoblast differentiation, such as osteocalcin, are poorly expressed or not present in the MET-OS clones, as they are in most human osteosarcomas.52 Therefore, our data show that the target cells of *MET*-driven transformation of the MET-OS clones were early committed osteo-progenitors. Interestingly, the MET-OS clones still express cyclins and cyclin-dependent kinases, which are typical markers of proliferating cells. This reflects the possible maintenance or reacquisition of the proliferative ability by committed progenitor cells.

Our findings are in agreement with results obtained in mouse models. Indeed, in mice targeting *Rb* and *p53* deletion to dermal fibroblasts results in the development of spindle cells/pleiomorphic sarcoma,53 the inactivation of the same genes in osteoblast precursors gives rise to osteosarcomas.12, 54 It has been shown recently that in mice, *Rb* loss impairs the osteoblastic differentiation of MSCs.36 This is apparently in contrast with the frequent *RB1* loss in human osteosarcomagenesis,10, 11 and with our finding of RB1 suppression in the MET-OS clones versus HOBs. However, as proposed by Calo and colleagues,36 both of these findings are in agreement with the hypothesis that osteosarcomas arise from committed osteo-progenitor cells and not from uncommitted MSCs. In this setting, *RB1* loss would allow de-differentiation, and thereby synergize with other mutations to promote tumorigenesis. In agreement, the MET-OS clones express markers of stemness, including *POU5F1*, *ABCB1* (*OCT3/4* and *MDR1*, respectively), and *CD117*. Both *CD117*15 and *OCT3/4* have already been identified as potential markers of osteosarcoma-initiating cells.13 Because we show that *MET* overexpression transforms osteoblast precursors rather than MSCs, the data presented here suggest that *MET*-driven osteo-progenitor amplification favors acquisition of either RB1 or p53 mutations in the progression of human osteosarcomas. Alternatively, *MET* overexpression in cells carrying RB1 or p53 mutations might sustain their "freezing" in a state of committed osteo-progenitors.

A clear role has not been defined for HGF and its receptor, MET, in osteoblast differentiation. The inconsistent data in the literature support both a pro-osteogenic role of HGF and the MET receptor,44, 45 and an anti-osteogenic potential.43 Because HGF and MET have been shown to stimulate proliferation of MSC-like cells,44, 55 it is conceivable that *MET* activation can have opposing roles that depend on the differentiation stage of the target cells. Here, we have shown that *MET* expression blocks osteoblast differentiation, and that MET kinase inhibition allowed the cells to acquire a more mature osteoblast phenotype. These findings further support the hypothesis that MET overexpression transforms osteo-progenitors rather than undifferentiated MSCs. MET kinase inhibition did not kill the MET-OS clones nor block cell proliferation as a whole. This suggests that the proliferation of MET-OS cells is independent of MET, but depends on other genes (see also 26). Conversely, MET inhibition abrogated the ability of the cells to form spheres, and their in vivo tumorigenesis. This finding suggests that

MET might contribute to osteosarcomagenesis by allowing transformed osteo-progenitors to maintain their self-renewal ability.

Altogether, the comparison between some osteosarcoma cell lines and MET-OS clones suggests that the latter are similar to a defined, possibly small, sub-group of human osteosarcomas, although all cell lines express the MET oncogene at a different extent. Indeed, osteosarcoma is a classification which includes tumors with likely varying genetic makeup.56 However, our model might contribute to the understanding of the definition of the role of *MET* in osteosarcomagenesis, which is important in view of *MET*-targeted therapies. *MET* has been identified as one of the most promising targets for molecular therapy, and several clinical trials are ongoing to test the numerous inhibitory molecules that have been developed (for reviews see 46, 47). *MET* has been shown to be overexpressed in more than 80% of human osteosarcoma samples.3–7 Altogether, the data shown here suggest that MET is not only important in the first step of osteosarcomagenesis, where it contributes to the expansion of a progenitor cell population, but also in the maintenance of the self-renewal ability and the inhibition of differentiation. It would be expected that the further progression of osteosarcoma can follow diverse pathways. Indeed, we show here that *MET*-transformed osteo-progenitors show expression profiles that are superimposable on those of some osteosarcoma cell lines, although all of the cell lines overexpress the *MET* oncogene.

CONCLUSIONS

This work shows that in osteosarcomagenesis *MET* might transform a cell population that has features of osteo-progenitors, which lack multilineage potential, that is, a cell population that in principle cannot self-renew. However, the MET-OS clones have self-renewal abilities, which are hampered by MET inhibitors. Therefore, although osteosarcomagenesis is completed by additional genetic events, it could be initiated and sustained by the activated *MET* oncogene and, thus *MET* might be an ideal target for osteosarcoma therapy.

Disclosures

All authors state that they have no conflicts of interest. The authors disclose any restrictions on access to raw data or statistical analyses.

Acknowledgements

We are grateful to Dr Antonino Sottile for the calcium content measurements. This work was supported by grants of the Italian Association for Cancer Research (AIRC) to MFDR and KS, Regione Piemonte (Ricerca Applicata) to MFDR, Compagnia di San Paolo to RF, Rete

Oncologica del Piemonte e della Valle d'Aosta and MIUR 2005 to FF, and Ministero della Salute (RF-IOR-2006-422755) to KS. Requests for materials should be addressed to Maria Flavia Di Renzo, MD, Laboratory of Cancer Genetics Institute for Cancer Research @Candiolo (IRCC); University of Torino School of Medicine; 10060 Candiolo; Turin, Italy. E-mail: mariaflavia.direnzo@unito.it

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