MET Overexpression Turns Human Primary Osteoblasts into Osteosarcomas

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Abstract

The *MET* oncogene was causally involved in the pathogenesis of a rare tumor, i.e., the papillary renal cell carcinoma, in which activating mutations, either germline or somatic, were identified. *MET* activating mutations are rarely found in other human tumors, whereas at higher frequencies, *MET* is amplified and/or overexpressed in sporadic tumors of specific histotypes, including osteosarcoma. In this work, we provide experimental evidence that overexpression of the *MET* oncogene causes and sustains the full-blown transformation of osteoblasts. Overexpression of *MET*, obtained by lentiviral vector–mediated gene transfer, resulted in the conversion of primary human osteoblasts into osteosarcoma cells, displaying the transformed phenotype *in vitro* and the distinguishing features of human osteosarcomas *in vivo*. These included atypical nuclei, aberrant mitoses, production of alkaline phosphatase, secretion of osteoid extracellular matrix, and striking neovascularization. Although with a lower tumorigenicity, this phenotype was superimposable to that observed after transfer of the *MET* gene activated by mutation. Both transformation and tumorigenesis were fully abrogated when *MET* expression was quenched by short-hairpin RNA or when signaling was impaired by a dominant-negative MET receptor. These data show that *MET* overexpression is oncogenic and that it is essential for the maintenance of the cancer phenotype. (Cancer Res 2006; 66(9): 4750-7)

Introduction

The *MET* oncogene encodes the tyrosine kinase receptor for the hepatocyte growth factor (HGF), which *in vitro* and *in vivo* elicits a unique physiologic program leading to morphogenesis, known as "invasive growth" (1). If deregulated, the invasive growth program contributes to cell transformation and tumor progression (2). The link between the *MET* oncogene and cancer has been unequivocally established after the identification of mutations in families suffering from a rare familial cancer, i.e., the hereditary papillary renal cell carcinoma (3). Thus far, *MET* mutations were also found in <20% of sporadic papillary renal cell carcinoma (4) and, at even lower frequency, in other human primary tumors (5–9) and metastases (7). Conversely, at higher frequencies, the *MET* oncogene is amplified and/or overexpressed in sporadic human tumors of specific histotypes (2). *MET* overexpression is associated with gene amplification in renal (10, 11), gastric (12), and colorectal cancers (13), and in gliomas (14). In other cancers, *MET* overexpression is attained by mechanisms other than gene amplification. For instance, other oncogenes, such as activated *RAS* (15), can induce *MET* overexpression, and increased transcription might also be caused by hypoxia (16).

The receptor encoded by the *MET* oncogene is expressed mainly in epithelial cells, whereas its ligand HGF is normally produced and secreted by cells of mesenchymal origin, suggesting that this ligand/receptor couple represents a paracrine signaling system for the mesenchymal-epithelial interaction in physiologic and pathologic conditions. Although barely detectable in adult tissues derived from the mesenchyme, during development, the MET receptor is expressed and has a nonredundant role in the migration of myogenic precursors (17), and possibly contributes to growth and/or survival of blood cells (18).

It is noteworthy that the *MET* proto-oncogene was originally identified as a transforming oncogene in a human osteosarcoma cell line (MNNG-HOS), which had acquired tumorigenicity after treatment with a chemical carcinogen (19). We and others showed that *MET* is misexpressed in mesenchymal tumors and that the highest levels of the receptor are detected in human osteosarcomas (>80%) in which *MET* expression correlates with an aggressive phenotype and poor prognosis (20–23). Therefore, we studied the contribution of MET receptor overexpression and activation in the transformation of human osteoblasts. In this work, we show that overexpression of the *MET* oncogene, at levels mimicking those found in human osteosarcomas, drives the conversion of human primary cultured osteoblasts into osteosarcoma cells, and is essential for the persistence of the transformed phenotype.

Materials and Methods

Human primary osteoblasts, tissue samples, and cell lines. Primary cultures of human osteoblast-like cells (HOB) were harvested from skull and appendicular bones of 6-month to 65-year-old patients treated for nonneoplastic diseases. Thirteen preparations were harvested from bone obtained from the pathologists, and consisted mostly of trabecular bone. As controls, two osteoblast preparations were purchased from PromoCell, Heidelberg, Germany. Fragments were repeatedly washed in PBS and collagenase at low concentrations to remove bone marrow, and then treated with 0.25% collagenase (type I, Sigma, St. Louis, MO) for 2 hours at 37°C. Outgrowing cells were cultured in DMEM with 10% fetal calf serum. Alkaline phosphatase content was determined on cell cytospins by

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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a cytochemical method (86R, Sigma). To analyze the osteogenic potential of HOB cells, cultures were also separately maintained for 3 weeks in differentiating medium supplemented with β-glycerophosphate (10 mmol/L), ascorbic acid (50 μg/mL), and dexamethasone (10^{-8} mol/L). The formation of mineralized calcium phosphate deposits was estimated by histochemical assay using alizarin red staining. Samples of human normal and tumor tissues were harvested and snap-frozen at surgery by the pathologists and stored at −80°C until RNA and protein extraction. The GTL-16 gastric carcinoma cell line has been previously described (24). The other cell lines were purchased from the American Type Culture Collection (Manassas, VA). Anchorage-independent growth was determined in 0.3% agarose (SeaPlaque; FMC Bioproducts, Rockland, MA) after 21 days culture. Colonies stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL, Sigma) were scored by two observers from three plates.

Lentiviral vector production and transduction. Replication-defective lentiviral vectors (LV) were generated by transiently transfecting 293-T cells with three separate plasmids. We used self-regulated LV carrying the expression cassettes for the tet-transactivator and for the transgene of interest within a single backbone (25) and bicistronic vectors, with the internal ribosome entry site of the encephalomyocarditis virus. The plasmids used included, the second-generation packaging construct which encodes the HIV-1 Gag and Pol precursors, as well as the regulatory proteins, Tat and Rev (pCMVΔR8.74), the VSV-G expressing construct, pMD.G, and the appropriate transfer construct. Dominant-negative MET receptor (DN-MET) vectors were generated as previously described (26). *MET*-specific short-hairpin RNA (MET-shRNA) sequences are as reported (16). We used the lentiviral transfer vector containing the polypurine tract of the pol sequences that acts in cis and enhances nuclear import and reverse transcription (25) in one transcriptional unit. Packaging 293-T cells were seeded in 10-cm diameter dishes 24 hours prior to transfection in Iscove's modified Dulbecco culture medium (JRH Biosciences, Inc., Lenexa, KS) with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL). Serial dilutions of freshly harvested vector stocks were used to infect 10^5 cells in a six-well plate in the presence of Polybrene (8 μg/mL). The viral p24 antigen concentration was determined by HIV-1 p24 Core profile ELISA (Perkin Elmer/NEN, Wellesley, MA) to determine the amount of infective particles before transduction and to show that transduced cells did not produce viral particles after transduction. Short-term cultures of HOB (from two to six population doublings) were transduced.

Southern and Northern blot analyses, end-point and real-time reverse transcription-PCR. Analyses were carried out as previously described (7). End-point reverse transcription-PCR for the detection of *MET*- and *HGF*-specific mRNA was carried out as described (7, 20). Quantitative "real-time" reverse transcription-PCR with TaqMan detection (ABI Prism 7700 Sequence Detection System, Perkin-Elmer Applied Biosystems, Foster City, CA) for the measurement of wild-type and mutant *MET*-specific transcripts were carried out as described (7).

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analysis to detect MET protein and tyrosine phosphorylated proteins were carried out as described (27). Polyclonal MET antibody (C-12, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), used for Western blot analysis, was raised against a peptide corresponding to 12 COOH-terminal amino acids of the c-*MET* human sequence; monoclonal antibodies DO-24 and DL-21 were used for immunohistochemistry and immunoprecipitation, and Western blotting, respectively, are directed against the extracellular domain of the MET protein.

Invasion assays in Matrigel and in three-dimensional collagen matrix. Matrigel invasion assay was done as described (27). To perform three-dimensional collagen matrix invasion assays, spheroids were generated as follows: 600 to 800 cells were suspended in culture medium containing 20% FCS and 0.24% methylcellulose (M-0512, Sigma), and seeded in round-bottomed 96-well plates. After overnight incubation at 37°C in a humidified atmosphere, all suspended cells contribute to the formation of a single spheroid. Thirty-six spheroids were harvested in each test tube, centrifuged for 15 minutes at 300 × *g* and resuspended in M-199 medium containing 0.696 μg/μL Rat Tail Collagene, type I (BD Biosciences, San Jose, CA), 0.016% methylcellulose, and 20% FCS. The spheroid-containing gel was rapidly transferred into each well of a prewarmed 96-well plate and allowed to polymerize for 1 minute. Fresh M-199 medium was added on gel tops and gels were incubated at 37°C in 5% CO_2 at 100% humidity and inspected for 48 hours. Each experiment was repeated at least thrice with identical results.

Tumorigenicity assay. Female 5- to 7-week-old severe combined immunodeficiency (SCID) mice (Charles River Italia, Como, Italy) were cared for in accordance with standards of the Italian law. Tumorigenicity was determined after s.c. injection in the ventral flank. Tumor growth was assessed twice weekly and mice were sacrificed 2 to 6 months after injection. Each tumor was divided into two: one-half was frozen in the presence of a cryopreservant or formalin-fixed and paraffin-embedded for routine histopathologic examination and immunohistochemistry. The other half was snap-frozen in liquid nitrogen and stored at −80°C until the time of protein, RNA, and DNA isolation. Western blot analysis, reverse transcription-PCR, and Southern blot analysis were carried out as above. Routine histopathology and immunohistochemistry for the detection of human MET and vimentin proteins were done according to standard protocols. A mouse anti-human vimentin monoclonal antibody that does not cross-react with mouse protein was used (V9, 1:100; Santa Cruz Biotechnology). A biotinylated secondary monoclonal antibody and Dab+ substrate chromogen system were used (Dako, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin. Specimens in which the incubation with the primary antibody was omitted were used as negative controls.

Results

***MET* oncogene overexpression transforms primary cultured human osteoblasts.** HOB preparations were propagated from 15 donors as monolayer cultures, and then characterized. HOB preparations were used in experiments when >80% of cells displayed the osteoblastic phenotype (Supplementary Fig. S1). As expected, in these cultures, the MET receptor was undetectable, as its expression is likely confined to more immature precursors (28) or is induced by hormones (29).

To obtain stable and sustained overexpression of the *MET* oncogene, multiple *MET* transgene copies were integrated into the HOB genome by means of a bicistronic LV. In this vector, the cytomegalovirus promoter powered the concurrent expression of *MET* and of a reporter gene (*GFP*). HOB cultures were transduced with either wild-type *MET* (MET^{wt}), or a mutated *MET* (MET^{Y1253D}), carrying the activating mutation Y1253D (7). After 40 to 60 days, stacks of brightly fluorescent cells had overgrown the dark monolayer of flat, large, and slowly growing cells (Fig. 1*A*). These stacks corresponded to the multilayered mounds of rapidly dividing cells that are usually described as "foci of transformation."

Individual foci were picked up and propagated as monolayer cultures, which showed unlimited growth in culture (see below). Cells of each focus-derived clone had an average of five copies of the bicistronic transgene integrated at distinct sites (Fig. 1*B*). Cells harvested from monolayers outside the foci of transformation were found to contain the *MET* transgene, randomly integrated (mean, 0.8 copies/cell; data not shown). Nonfluorescent foci were never found. Foci did not develop in control HOB cultures or in cultures transduced with control LVs (carrying either no transgene, GFP transgene alone, or bicistronic luciferase-GFP cassette; data not shown). MET receptors were only detected in foci-derived clones, as shown by Western blot analysis (Fig. 1*D*) or immunohistochemistry (Supplementary Fig. S2*A*). In individual clones, levels of MET protein overexpression paralleled the levels of *MET* mRNA (Table 1). The overexpressed MET receptors were in a constitutively activated state, as shown by autophosphorylation at tyrosine (Fig. 1*C*). The mutant MET^{Y1253D} receptors showed a slightly lower

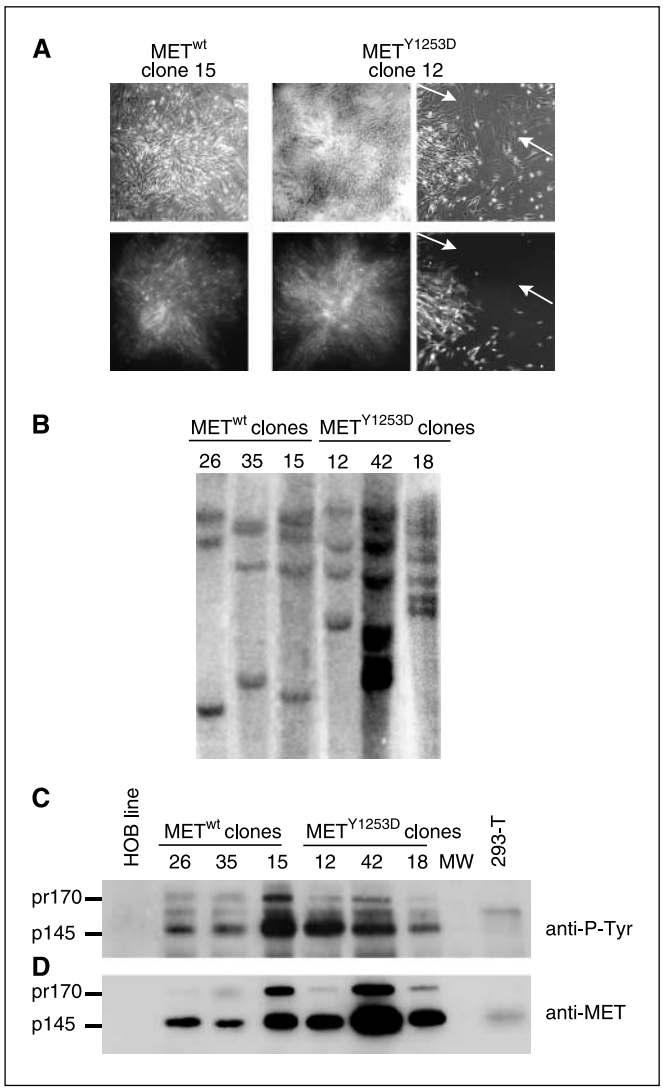

Figure 1. *MET* transgene expression and activation in HOB clones. HOB preparations were transduced with bicistronic LVs carrying the *MET*-internal ribosome entry site-*GFP* cassette with either the wild-type (MET^{wt}) or the mutated (MET^{Y1253D}) *MET* cDNA. Results of the transduction of the HOB preparation no. 1704. *A,* after 40 to 60 days, a similar number of foci (35-45 each/10-cm diameter culture dish) developed in MET^{wt} (*left*) and in MET^{Y1253D} (*middle* and *right*) HOB cultures. Foci of birefringent (*top*) and strongly fluorescent (*bottom*) cells overgrew a monolayer of flat and nonfluorescent cells, which were visible at higher magnification (*arrows*). *B,* Southern blot analysis of DNA from cells propagated from individual foci showed stable integration of the transgenes. Numbers on top indicate different clones. The blot was labeled with a probe specific for the *GFP* gene. DNA was digested with *Spe*I. As the *Spe*I site is unique in the vector, the labeling of discrete different bands in different foci showed the random integration of the transgene in HOB cells and the clonality of foci. Activation (*C*) and expression (*D*) of the MET receptor in clones. Proteins immunoprecipitated by anti-MET monoclonal antibodies were labeled in Western blot analysis with a monoclonal antibody against phosphotyrosine (*C*) and subsequently with MET monoclonal antibody against the human COOH terminal peptide of the receptor (*D*). Proteins were separated in the presence of a reducing agent, which takes apart the α- and β-chains of the MET receptor. Antibodies labeled the MET β-chain (p145) and the MET precursor (pr170). As positive controls proteins from 293-T cells, which express the MET receptor (see also Table 1), were loaded. The receptor was not found in parental HOB cultures.

level of phosphorylation than the wild-type ones, as expected, due to the substitution of one of the major MET phosphorylation sites (30) with an aspartic acid. This did not impair its kinase activity that was even increased by the negative charge mimicking the phosphorylated tyrosine; these data are not shown because they were reported elsewhere (7). We tested whether any of the *MET*-overexpressing HOB clones produce the MET ligand HGF, using both reverse transcription-PCR with specific primers and Western blot analysis (data not shown). Although in these cells, the MET receptor was in a constitutively activated state, in no instance was HGF detectable either as a protein or mRNA. This ruled out the idea that MET receptor activation was due to an autocrine loop.

Transformed human osteoblasts express *MET* levels comparable to that of human osteosarcomas. Using quantitative reverse transcription-PCR, we compared the level of *MET* expression with that of human cancer cell lines and bioptic samples (Table 1). In HOB clones, the level of *MET* expression was in the range measured in gastric and ovarian carcinoma and osteosarcoma cell lines, already classified as *MET*-overexpressing cell lines. Similar amounts of *MET*-specific mRNA were found in spontaneously occurring osteosarcomas and other human tumors.

Although the level of *MET* expression attained in HOBs was comparable to that of human osteosarcomas, we hypothesize that only HOBs expressing *MET* over a critical threshold acquired the transformed and immortalized phenotype. To verify this hypothesis, we expressed the MET receptor in HOBs at levels comparable to those observed in physiologic conditions in human tissues (13, 31), using a tet-dependent self-regulating LV (25). In this case, *MET*-transduced HOB cultures were indistinguishable from those of parental HOBs. Foci did not develop in these cultures which, however, showed the ability to form colonies in semisolid medium at high efficiency, as long as they expressed the MET receptor (Fig. 2). These cultures underwent growth arrest like the parental HOBs after 40 to 70 days, suggesting that the neoexpression of the MET receptor at physiologic levels is able to confer clonogenic ability to human primary osteoblasts, but not immortalization.

***MET*-overexpressing human osteoblasts show a fully transformed phenotype *in vitro* and tumorigenicity *in vivo*.** The *MET*-overexpressing HOBs propagated from foci-derived clones showed a fully transformed phenotype *in vitro* and were tumorigenic *in vivo*, showing an osteosarcoma-like phenotype.

The *MET*-overexpressing HOBs showed anchorage-independent growth, whereas the parental HOB did not (Table 2). Nevertheless, clones maintained the osteogenic phenotype, e.g., production of alkaline phosphatase and mineralized matrix (Supplementary Fig. S2*B* and *C*; data not shown). Supplementary Fig. S2 and Table 2 show that cells propagated from foci of transformation, when grown as monolayer cultures, were spindle-shaped and birefringent, whereas the parental HOBs were cuboidal and flat, did not show contact inhibition, showed anchorage-independent growth, displayed high mitotic activity, and exhibited unlimited growth.

In vitro and *in vivo*, the MET receptor activated by its ligand HGF triggers a unique biological program leading to the so-called "invasive growth" (1). Deregulated activation of the MET-mediated "invasive-growth" program has long been thought to contribute to cell transformation and tumor progression (2). We found that the *MET*-overexpressing HOBs displayed biological properties that recapitulate the activities elicited by MET signaling *in vitro*. They acquired the ability to invade an artificial basement membrane made of collagen, laminin, and glycosaminoglycans (Matrigel), whereas parental osteoblasts did not (Supplementary Fig. S3*A*). This chemoinvasion assay is commonly used to evaluate cancer cell invasiveness. These cells also showed the ability to invade a three-dimensional collagen gel (Supplementary Fig. S3*B*). This assay, also known as the "branching morphogenesis" assay, highlights the

potential of cells to invade a surrogate extracellular matrix, forming typical branched structures. This invasion process represents the summa of the invasive growth phenotype and results from the fine integration of all the pleiotropic effects induced by HGF, including cell proliferation, motility, differentiation, and survival (16).

We found that the *MET*-overexpressing HOB clones had complex cytogenetic and chromosomal aberrations. For example, in a wild-type *MET*-overexpressing clone, we found 84% of hyper-diploid cells (mean chromosome number, 52 ± 5) and 6% of hyper-tetraploid cells. In a MET^{Y1253D}-overexpressing clone, we found 83% of hyper-diploid cells (mean chromosome number, 54 ± 1) and 7% of hyper-tetraploid cells. In addition, only a few chromosomes were recognizable, as many were rearranged. However, common aberrations were not detected (data not shown). It is noteworthy that both the parental osteoblasts and osteoblasts transduced with control vectors were diploid and devoid of aberrations as long as they were propagated in culture. The genetic alterations of *MET*-overexpressing HOB clones are reminiscent of the karyotypes of human osteosarcoma (32), which is characterized by complex chromosomal abnormalities, often with pronounced cell-to-cell variation or heterogeneity, and, in contrast with other human sarcomas, is not associated with any specifically recurrent translocation or any other specific chromosomal rearrangement (33).

The *MET*-overexpressing HOB clones were tumorigenic *in vivo* (Fig. 3). As expected, due to the known low growth efficiency of human osteosarcoma cells in SCID mice (34), a fairly high number of transplanted cells was necessary to obtain xenografts, and a low incidence was observed (Table 3). All tumors showed the distinguishing features of highly aggressive human osteosarcomas (35), including atypical nuclei and aberrant mitoses (Fig. 3), the production of some osteoid extracellular matrix and alkaline phosphatase, the expression of human vimentin, and striking neovascularization (data not shown). Figure 3 also shows that all tumors expressed the *MET* transgene mRNA and the human MET receptors detectable by immunohistochemistry.

As mentioned in the first paragraph, we also obtained HOBs overexpressing a *MET* oncogene carrying the Y1253D activating mutation (7). As shown in Supplementary Figs. S1 and S2, HOB clones overexpressing the mutated MET^{Y1253D} were phenotypically

Table 1. *MET* expression level in HOB clones and human tumors

Cells	Tissue	Transgene*	MET^{wt} expression (C_T)†	MET^{Y1253D} expression (C_T)†
HOBs				
Parental		None	>40	>40
Clone 15	Osteoblasts	MET^{wt}	20.1	>40
Clone 26	Osteoblasts	MET^{wt}	22.0	>40
Clone 35	Osteoblasts	MET^{wt}	22.4	>40
Clone 12	Osteoblasts	MET^{Y1253D}	Not tested	21.0
Clone 18	Osteoblasts	MET^{Y1253D}	Not tested	20.2
Clone 42	Osteoblasts	MET^{Y1253D}	Not tested	19.0
Cell lines				
293-T	Embryonal kidney	None	28.2	>40
GTL-16	Gastric carcinoma	None	19.0	>40
SK-OV-3	Ovarian carcinoma	None	22.3	>40
NIHOVCAR3	Ovarian carcinoma	None	21.0	>40
U2-OS	Osteosarcoma	None	25.2	>40
SAOS-2	Osteosarcoma	None	23.9	>40
MG-63	Osteosarcoma	None	24.7	>40
Samples ‡				
OS 1	Osteosarcoma	None	21.7	>40
OS 2	Osteosarcoma	None	22.3	>40
OS 3	Osteosarcoma	None	24.4	>40
NL 95	Normal lymph node	None	>40	>40
NL 106	Normal lymph node	None	>40	>40
HN mucosa 95	Squamous epithelium	None	29.7	>40
HN mucosa 106	Squamous epithelium	None	31.5	>40
HNSCC-T65	Squamous cell carcinoma	None	27.0	33.5§
HNSCC-M65	Squamous cell carcinoma metastasis	None	22.5	24.4§
HNSCC-T66	Squamous cell carcinoma	None	27.7	31.6§
HNSCC-M65	Squamous cell carcinoma metastasis	None	23.7	23.0§
OVCAR H01	Ovarian carcinoma	None	21.0	>40
OVCAR E12	Ovarian carcinoma	None	22.3	>40
OVCAR A8	Ovarian carcinoma	None	21.9	>40

*Genes transduced by LVs.

†*MET* transgene expression was measured by quantitative real-time RT-PCR with TaqMan assay. C_T (threshold cycle) indicates the number of cycles needed to reach a threshold amount of PCR products and depends directly on the initial concentration of target nucleic acid; >40, not detectable.

‡HNSCC, head and neck squamous cell carcinomas.

§See ref. 7.

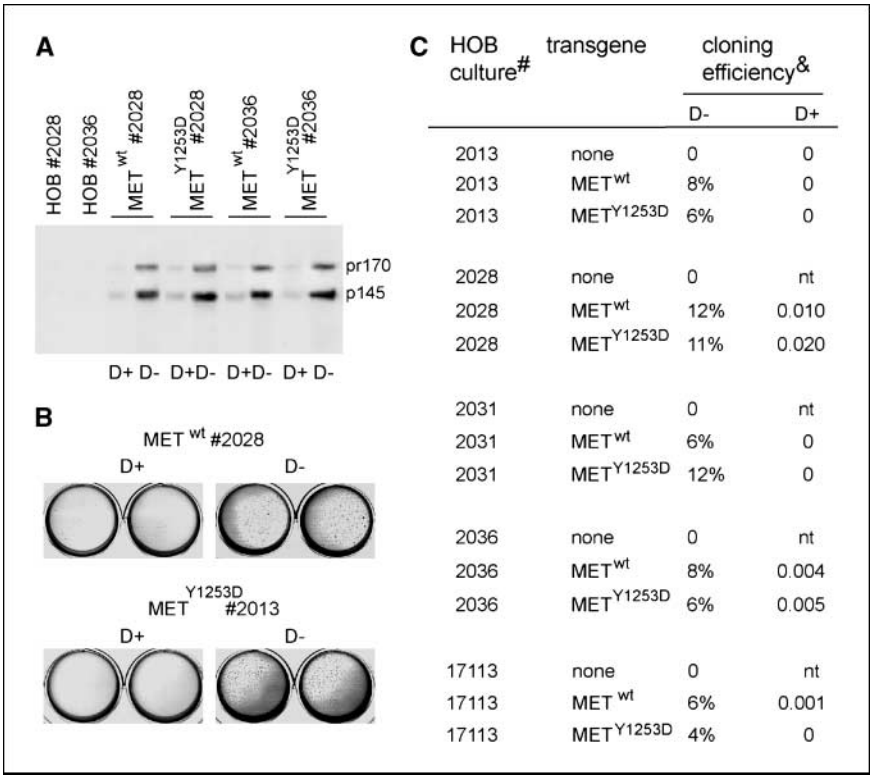

Figure 2. *MET* transgene regulated expression and biological outcome in HOB cultures. *A,* HOB cultures were transduced with either the wild-type (MET^{wt}) or mutated (MET^{Y1253D}) *MET* transgenes using tet-self-regulated LVs. The bulk of the unselected populations of transduced HOBs were studied. When grown in the absence of doxycyclin (*D*−), HOBs expressed detectable MET receptors, which decreased on the addition of doxycyclin (*D*+). In parental HOB cultures (no. 2036 and no. 2028), the MET receptor was never found. Total proteins were extracted from the bulk unselected cell populations and analyzed in Western blot using MET monoclonal antibodies against the human COOH terminal peptide. Proteins were extracted to allow the separation of the α- and β-chains of the MET receptor. Antibodies labeled the MET β-chain (p145) and the MET precursor (pr170). *B,* HOB cultures expressing either MET^{wt} or MET^{Y1253D} transgene formed visible colonies in semisolid agar medium in the absence (*D*−) but not in the presence (*D*+) of doxycyclin. *C,* colonies formed by different HOB cultures transduced with either the MET^{wt} or the MET^{Y1253D} transgene were stained and counted under the microscope: *#,* different HOB preparations from different donors; &, colonies were counted 21 days after plating 1×10^3 and 4×10^3 cells in triplicate; the percentage of cells which formed colonies out of those plated are the mean of three plates in a representative experiment; *nt,* not tested.

indistinguishable from HOBs overexpressing the wild-type *MET*, the notable exception being the lower *in vivo* tumorigenicity (Table 3).

***MET* "addiction" of human osteoblasts transformed by MET.** We next evaluated if the transformed and tumorigenic phenotype displayed by *MET*-overexpressing HOB clones relied on sustained MET receptor activation. Activation was almost abolished (Supplementary Fig. S4) after either decreasing MET receptors by stable expression of MET-shRNA or by blocking MET receptor dimerization by stable expression of a DN-MET. The latter lacks the intracellular domain and thus forms inactive dimers with the full size receptor (26). In both cases, the transformed phenotype, measured as the ability to undergo anchorage-independent growth, was consistently reduced (Table 2), and the invasion assay *in vitro* scored negative (Supplementary Fig. S3*B*). *In vivo*, the stable expression of the DN-MET receptor abolished the tumorigenic properties of *MET*-overexpressing clones (Table 3). The DN-MET was also expressed in established human osteosarcoma cell lines, where we previously showed *MET* overexpression and constitutive activation [ref. (20); Supplementary Fig. S5]. The

Table 2. Proliferation of *MET*-overexpressing HOB clones

Cells	Transgene*	Cloning efficiency in soft agar (%)†	PD‡ after 180 days
Parental HOBs	None	0	Senescent after 40-70 days (15-25 PD)
Clone 15	MET^{wt}	18.0	140
Clone 15 DN-MET	$MET^{wt,\ DN\text{-}MET}$	3.6	120
Clone 15 shRNA	$MET^{wt,\ shRNA}$	3.3	120
Clone 26	MET^{wt}	17.5	140
Clone 35	MET^{wt}	9.5	Not tested
Clone 12	MET^{Y1253D}	16.5	150
Clone 12 DN	$MET^{Y1253D,\ DN\text{-}MET}$	7.0	130
Clone 12 shRNA	$MET^{Y1253D,\ shRNA}$	5.0	130
Clone 18	MET^{Y1253D}	5	115
Clone 42	MET^{Y1253D}	28.0	125
Clone 42 DN	$MET^{Y1253D,\ DN\text{-}MET}$	7.2	120
Clone 42 shRNA	$MET^{Y1253D,\ shRNA}$	3.3	120

*Genes transduced by LVs.
†Cell colonies were counted 21 days after plating 1×10^3 and 4×10^3 cells in triplicate.
‡PD, population doublings: in clones, the stability of transgene integration and expression was monitored every 20 PD.

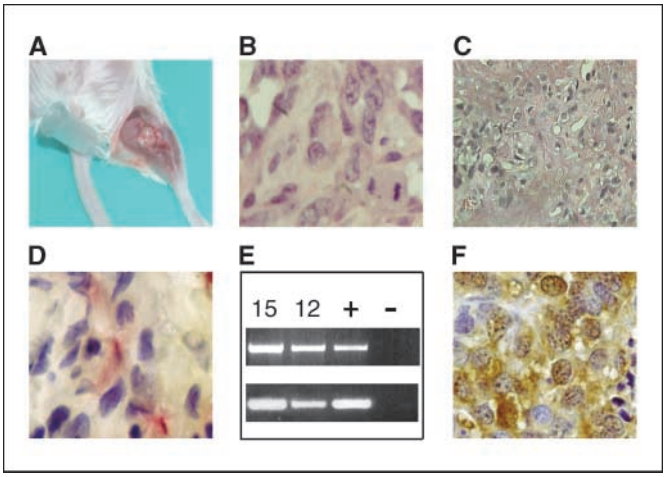

Figure 3. Tumorigenic properties of HOB clones overexpressing the MET^{wt} or the MET^{Y1253D} receptor. *A,* tumorigenic ability was measured in immunocompromised SCID mice. *B-F,* phenotypic characterization of tumors grown after s.c. injection. Tumors had the histology of a highly aggressive osteosarcoma, with several atypical nuclei and aberrant mitoses (*B*). Although mostly undifferentiated, tumors showed areas of osteoid production (*C*) and alkaline phosphatase expression (*D*). Tumors carried integrated copies of the transgene (data not shown); all expressed the relevant transgene. *E,* expression of the transgene in the propagated clones no. 12 and 15 (*top*) and in the corresponding xenograft (*bottom*); +, positive (GTL-16 cells) controls; −, negative (HOB parental cultures) controls. *F,* immunohistochemical detection of the MET receptors in a tumor with antibody against the human COOH terminal peptide of the receptor.

DN-MET fully abolished MET constitutive activation (Supplementary Fig. S5) and, intriguingly, also impaired their growth *in vivo* as xenografts (Table 3).

Discussion

In this work, we show that the overexpression of the *MET* oncogene initiates the transformation of human primary osteoblasts and sustains tumor progression towards the osteosarcoma.

We obtained overexpression by transferring the wild-type *MET* transgene by means of LVs, which ensures the random integration of the *MET* transgene in the majority of cultured cells, as LVs are also able to transduce nondividing cells. The presence in each *MET*-overexpressing clone of the transgene integrated at distinct sites showed that individual foci originated from random and different integration events, and thus makes the contribution to transformation of activation of proto-oncogenes or inactivation of tumor suppressor genes as an unlikely consequence of vector insertions. The former was per se improbable, as we used LV with a self-inactivating LTR (36). Random integration also allowed us to rule out the role of integration sites on expression variation. Therefore, transgene expression only depends on its intrinsic regulation, which relies on the internal promoter. In addition, a particular advantage of transgene expression operated by LV gene transfer is a constant linear relationship between the number of integrated copies and amount of mRNA (36). We also show that foci of transformation did not develop in control osteoblast cultures or in cultures transduced with control LVs. These data strengthen the established notion that spontaneous transformation of *in vitro* cultured human cells is an infrequent event (37), and thus, rule out the unlikely possibility that we selected transformed cells arising independently from *MET* expression.

In vitro, the HOB clones overexpressing the wild-type *MET* display the same properties of those transformed by the *MET* oncogene activated by a known mutation. This was not surprising, as we found that *in vitro*, the overexpressed wild-type receptors are constitutively active, even in the absence of the ligand HGF, likely due to self-oligomerization (38). *In vivo*, HOB clones overexpressing the mutated MET receptors were more tumorigenic. It is conceivable that the threshold to activate the *in vivo* growth might be higher and more easily overcome in the presence of some cross-reactive mouse HGF present in circulating blood. As it has been shown that some *MET* mutations sensitize the MET receptor to very low HGF concentrations (39), we can speculate that the MET^{Y1253D} receptor made osteoblasts more sensitive to mouse HGF.

In the transformed osteoblast clones, the level of *MET* expression was in the range measured in known overexpressing cell lines (24, 27, 40) and in human cancers (7, 31, 41), including osteosarcomas (20). Thus, the high level of expression, obtained by

Table 3. Tumorigenic properties of *MET*-overexpressing HOB clones

Cells	Transgene*	No. of mice with tumor / injected mice †	Latency (days)
Parental HOBs	None	0 of 8	—
Clone 15	MET^{wt}	3 of 8	100-135 (mean, 110)
Clone 12	MET^{Y1253D}	9 of 12	40-120 (mean, 55)
Clone 12 DN	$MET^{Y1253D,\ DN\text{-}MET}$	0 of 4	—
Clone 42	MET^{Y1253D}	6 of 8	50-70 (mean, 60)
Clone 42 DN	$MET^{Y1253D,\ DN\text{-}MET}$	0 of 4	—
U2-OS ‡	None	4 of 4	50-55 (mean 53)
U2-OS DN	DN-MET	0 of 4	—
IOR-OS9 ‡	None	4 of 4	37-70 (mean, 65)
IOR-OS9 DN	DN-MET	0 of 4	—

*Genes transduced by LV.

†Mice s.c. injected with 30×10^6 cells; clones overexpressing the MET^{Y1253D} transgene were more tumorigenic than the MET^{wt} expressing clones. The mean percentage ($\pm$2 SE) of mice with tumors out of mice injected with either MET^{wt} or the MET^{Y1253D}-expressing cells were 16.7 $\pm$ 12.5% and 75 $\pm$ 16.7%, respectively (t = 4.08; P < 0.05).

‡U2-OS and IOR-OS9 human osteosarcoma cell lines, as well as the MET^{Y1253D} HOB clones, were no longer able to form tumors in nude mice when transduced with the DN-MET transgene.

mimicking *MET* gene amplification using LV-mediated gene transfer, did not exceed that observed in spontaneously occurring osteosarcomas and other human tumors. We can conclude that osteoblast transformation and tumorigenesis are not the result of massive oncogene overexpression.

Human osteoblast transformation *in vitro* is a rare event. A likely hypothesis that explains the rarity is the number of susceptible target cells being very small. A candidate for this minor population could be the still elusive osteoprogenitor cell that maintains the potential to proliferate and to differentiate (42). Progenitors are indubitably present in primary osteoblast cultures, which undergo several population doublings before senescence (43), and previous work has shown that LVs transfer genes in bona fide stem cells (44), including bone precursors (45). Although unidentified, the osteoprogenitor targeted by *MET* likely belong to the family of mesenchymal stem cells, which are also the precursors of rhabdomyosarcomas. Interestingly, we (46) and others (47) found *MET* overexpression in a high percentage of human rhabdomyosarcoma; furthermore, in a transgenic mouse model, deregulated MET signaling contributes to rhabdomyosarcomagenesis (48). As we show here, *MET* misexpression alone does not transform, but it is able to confer clonogenic capability to osteoblasts. Altogether, these data suggest that the expression of *MET* oncogene creates an expanded premalignant osteoblast population; in this population, overexpression of the oncogene might either lead or simply allow the occurrence of other transforming events in mesenchyme-derived osteoblast progenitors, e.g., cancer stem cells, thus uniquely contributing to osteosarcomagenesis. In both instances, the transformed and tumorigenic phenotype relies on the continuous presence of an activated MET receptor.

This article provides conclusive experimental evidence supporting the long-sought link between *MET* overexpression, found in a large repertoire of human cancers, and progression toward malignancy (2). Moreover, these findings are consistent with the notion that increased copy number of the *MET* gene and its expression above a threshold are also critical requirements for the development of tumors harboring germ line *MET* mutations (10, 49). Interestingly, *MET* overexpression is required to sustain the fully blown osteosarcoma phenotype; both transformed osteoblasts and *MET*-overexpressing osteosarcoma cell lines showed *MET* "addiction." This information highlights *MET* as a therapeutic target in human cancers accumulating genetic alterations.

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