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***De novo* expression of EphA2 in osteosarcoma modulates activation of the mitogenic signaling pathway**

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Abstract

Aims: In osteosarcoma patients, the development of metastases, often to the lungs, is the most frequent cause of death. To improve this situation, a deeper understanding of the molecular mechanisms governing osteosarcoma development and dissemination and the identification of novel drug targets for an improved treatment are needed. **Methods and results:** Towards this aim, we characterized osteosarcoma samples using genome-wide microarrays and detected increased expression of the *EphA2* receptor and its ligand *EFNA1* in osteosarcoma tissues. In addition, increased expression of *EFNB1*, *EFNB3* and *EphA3* was suggested. Immunohistochemical staining revealed an absence of EphA2 from normal bone, and *de novo* expression in osteosarcomas. EFNA1 was expressed in normal bone, but significantly elevated in tumors. This pattern suggests a specific role for the EFNA1-EphA2-interaction in osteosarcoma. Further *in vitro* investigations on the functional role of EphA2 and EFNA1 showed that EFNA1 ligand binding induced increased tyrosine phosphorylation, receptor degradation and downstream MAPK activation. Interference with the MAPK pathway unraveled a potential autoregulatory loop governing mainly EFNA1 expression via the same pathway. **Conclusion:** Our results indicate that upregulation and *de novo* expression of ephrins in osteosarcomas are involved in oncogenic signaling and thus might stimulate osteosarcoma metastasis.

Introduction

Conventional osteosarcoma, also called classical osteosarcoma, is the most common malignant primary bone tumor in children and adolescents with a profound risk of metastasis to the lung¹. This highly aggressive neoplasm mainly affects patients in the second decade of life when the growth spurt is highest, with 60% of patients under the age of 25². Before the use of effective chemotherapy, the 2-year overall survival rates for patients with osteosarcomas was 15% to 20% following surgical resection and/or radiotherapy³. Surgery and chemotherapy since then led to an improvement in long-term survival rates to 50% to 80%⁴. Nevertheless, drug resistance and poor clinical outcome due to early metastasis are still a problem.

Using microarray analysis we recently detected several ephrins and their cognate receptors deregulated in human bone tumors such as giant cell tumors and chondrosarcomas⁵. Ephrin receptors (Eph) are the largest group of receptor tyrosine kinases, however to date most information about ephrin receptors and ephrin ligands (EFN) was gained from melanoma and epithelial tumors⁶. In mouse melanoma cells, expression of the EphA2 receptor was shown to stimulate a distinct invasion program resulting in metastasis formation⁷. In carcinomas, there is already ample information on the diverse roles of different EFN and Eph and their signaling⁸. In colorectal cancer, loss of the EphA1 protein has been correlated with invasion and metastasis and is therefore associated with a poor prognosis^{9, 10}. In contrast, EphA2 was found upregulated in many carcinomas and exhibits both a kinase-dependent and kinase independent role in the metastasis of prostate cancer¹¹. The research on ephrin involvement in tumor development and progression is complicated by the fact that ephrin receptors and their ligands display forward and reverse signaling, yet the impact of both EphA and B family members in angiogenesis is now well documented¹².

Based on current molecular knowledge, targeted and rational therapeutic strategies are developed and nowadays successfully improve survival of cancer patients. Nevertheless, there is a continued medical need to study the basic molecular mechanisms of osteosarcoma in order to develop bone tumor-specific therapeutic strategies. The current study aimed to identifying genes differentially expressed between normal human osteoblasts and osteosarcoma tissue and to define genes that may be involved in tumor progression. This analysis revealed a distinct ephrin expression pattern and an increased expression of EFNA1 ligand and EphA2 receptor in human osteosarcoma tissue.

Material and methods

Patients and tissues

Tumor samples were obtained from the Max-Delbrueck-Center, division of Pathology, Berlin-Buch, Germany. Patient characteristics are described in Tab. 1. The tissue specimens were fixed in 4% neutral buffered formaldehyde, bone containing tissue was EDTA-decalcified and embedded in paraffin. For each case, hematoxylin and eosin (H&E)-stained slides were carefully reviewed and the diagnosis of the tumor was confirmed according to WHO criteria. Written informed consent was obtained from each patient. The median age of osteosarcoma patients is 37 and ages vary from 7 years to 74 years. This is unusual as osteosarcoma typically affects adolescents, but we were not able to use younger patients based on the fact, that our sample collection in the institute include only these patients. All of the osteosarcoma tumors are primary.

Cell culture

Primary osteoblast cells (HOBc) and the osteosarcoma cell line SaOS2 were purchased from Provitro GmbH, Berlin, Germany and from ATCC (American Type Culture Collection) and

cultured in osteoblast growth medium (PromoCell, Heidelberg, Germany) and McCoys5A media (Gibco) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, respectively. The osteosarcoma cell lines HOS, MNNG/HOS, OST, SJSA, MG63 and ZK58 were a gift from Prof. Dr. G. Gaedicke, Clinic for General Pediatrics and Experimental Oncology, Charité Universitätsmedizin Berlin, Berlin, Germany and cultured in RPMI1640 (Biochrome) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. All cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C. For signaling pathway analyses 1µg/ml EFNA1/F_c (R&D), 50µM U0126 (Promega) or 50µM DMSO (Sigma) were used for the time points indicated. For proliferation assays cells were directly plated on 96-plates or 96-polyhema-plates and treated with 1µg/ml EFNA1/F_c up to 96h. For anchorage-independent growth media containing EFNA1/F_c was changed every 24h. Proliferation of cells was measured using the MTT cell proliferation kit (Roche). For wound healing assay, cells were grown on 6-Well dishes or glass slides as a confluent monolayer and wounded with a 10µl pipette tip. Afterwards, 1µg/ml EFNA1/F_c in the presence of fetal calf serum was added and wound closure was observed up to 48 hours.

Microarray analysis

All tissues were cryopreserved in liquid nitrogen immediately after removal and stored at -80°C. RNA was isolated from frozen samples and fresh cells using Tri-Reagent (Sigma) as well as the Rneasy-mini-kit (Qiagen). cRNA was synthesized from 5µg of total RNA. Synthesis of biotin-labeled cRNA was performed using the BioArray High Yield RNA Transcription kit (Enzo Diagnostics, Farmingdale, N.Y.). The amplified cRNA was hybridized to HG U133A arrays (Affymetrix, Santa Clara, CA). After a scan of the array surface, the computer-generated image of the array was overlaid with a virtual grid, controlled by Microarray Suite 5.0 software (Affymetrix). The Data Mining Tool 3.0 (Affymetrix) and GeneSpring software package 6.1 (Silicon Genetics, Redwood City, CA) were used to

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average results from different replicates and to perform statistical analysis to compare the sample groups. Differential gene expression between osteosarcoma and non-neoplastic osteoblasts was defined according to the following criteria: 1) upregulated genes are present in both replicates of osteosarcoma metastases and differential expression between conventional versus HOBc (>1.5) and metastases vs. HOBc (>1.5) is mandatory, 2) downregulated genes are present in both replicates of HOBc and a differential expression between conventional vs. HOBc (>1.5) and metastases vs. HOBc (>1.5) is mandatory. Expression levels below 1,000 were defined as background. The data were submitted to the GEO (Gene Expression Omnibus) database (GEO accession number GSE14359, geo@ncbi.nlm.nih.gov).

Immunohistochemistry

Immunohistochemical analyses were performed on paraffin-embedded tissues. As a non-neoplastic control, a tissue microarray (TMA) containing n=4 fetal and n=6 adult bone samples (each in duplicates; Provitro GmbH, Berlin, Germany) was used. Antigen retrieval was performed by incubation in trypsin (0.5%, Zymed Digest-All Kit). To block endogenous peroxidase activity, peroxidase blocking reagent (DAKO) was used. The primary antibodies EFNA1, EFNA2, EFNB1, EFNB3 (all 1:10, Zymed) and EphA2 (1:10, Upstate) were incubated over night at 4°C. The chromogene was 3, 3'-diaminobenzidine (DAB, DAKO) and nuclear counter staining was performed with hemalaun. All samples were semi-quantitatively scored by two different observers using a score modified according to *Remmele and Stegner*¹³. 1. A score to measure the percentage of positively stained cells (PP) was performed as follows: 0 = no staining, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 = 76-100%. 2. A staining intensity score (SI) was established to measure the intensity of positively stained cells: 0 = no expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression. The combined immunoreactivity score (IRS) value was calculated through PPxSI (maximum

value was 12). Statistical analyses were performed executing the Mann-Whitney-U-Test and Bonferroni correction p-values using SPSS software 13.0. Differences were considered significant at $p < 0.05$.

Immunoblot analysis

Protein extracts were prepared by incubation with sodiumdodecylsulfate (SDS)-cell lysis buffer (10% SDS, 1M TrisHCl pH 7.5, EDTA 0.5M pH8). Reagents for SDS-PAGE and Western blotting were obtained from Bio-Rad Laboratories (Richmond, CA). Electrophoresis was performed and proteins were transferred to nitrocellulose membranes (Schleicher & Schüll). Membranes were blocked and specific proteins were detected by incubation with rabbit anti-human EFNA1 (V-18, 1:200 Santa Cruz), mouse anti-human EphA2 (Clone D7, 1:500 Upstate), mouse anti-human p-Tyr (1:1,000 Santa Cruz), rabbit anti-human P-Erk1/2 (p44/42 MAPK Thr202/Tyr204 1:1,000 Cell Signaling), mouse anti-human Erk1 (1:5,000 BD) and mouse anti-human actin (1:5,000 Chemicon) antibodies over night at 4°C following HRP-conjugated rabbit anti-mouse (1:5,000 Dianova) or goat anti-rabbit (1:2,000 Cell Signaling) secondary antibodies. Blots were developed using ECL system from Amersham. The quantification of blots was done using ImageJ.

Immuofluorescence staining

Cells were grown on glass slides and fixed with acetone. Immunofluorescence staining was performed using mouse anti-human EphA2 (Clone D7, 1:10 Upstate) antibody and visualized with Alexa488 (1:300 Invitrogen). Nuclei were counterstained with DAPI. Images were recorded using a fluorescence microscope (Zeiss).

Results

Expression of ephrins in osteosarcomas

RNA derived from eight osteosarcoma samples (n=4 conventional, n=4 metastases) and from the human osteoblast primary culture HOBC were analyzed using Affymetrix HG U133A microarrays. 1,163 genes were found significantly upregulated in conventional and metastatic osteosarcomas as compared to HOBC. 31 genes were expressed >10 fold in osteosarcomas compared to the non-neoplastic osteoblasts. Among them, *EFNA1*, a ligand for EphA tyrosine kinase receptors, was expressed >10 fold higher in osteosarcoma samples compared to osteoblasts (Tab. 2A). In addition, 2,059 genes were significantly downregulated between the osteosarcomas and HOBC. 37 genes were expressed less than 10 fold in osteosarcoma samples compared to osteoblasts (Tab. 2B).

During a recent study we discovered several ephrin genes deregulated in bone-related tumors, such as giant cell tumor of the bone⁵. For example, *EFNA1* and *EphA3* were upregulated in recurrent as compared to primary giant cell tumors, while *EphA1* and *EphA4* were found downregulated in giant cell tumor recurrences. This prompted us to suggest that tissue-specific expression patterns of ephrins are associated with the development and progression of bone tumors. The observation that the *EFNA1* ligand light up as highly upregulated in the osteosarcoma samples versus non-neoplastic osteoblasts focused our further survey to the ephrin family and their expression in osteosarcoma tumor samples (Tab. 3). Within the 22,283 Affymetrix probe sets are five EFNA ligand probe sets (EFNA1-5), six EphA receptor probe sets (EphA1-5, EphA7), three EFNB ligand probe sets (EFNB1-3) and five EphB receptor probe sets (EphB1-4, EphB6).

EFNA1, *EphA2* and *EphA3* were found upregulated in osteosarcoma tissue compared to HOBC. No significant differences between conventional and metastases could be observed. *EFNA4*, *EphA1*, *EphA4* and *EphA5* were found to be expressed above background only in 1/8 osteosarcoma samples but below background in HOBC. Expression levels for *EFNA2*,

EFNA3, *EFNA5* and *EphA7* were below background. Among the B-ligands, only *EFNB2* showed a high expression in osteosarcomas and HOBC, whereas expression below background was observed for *EFNB1*, *EFNB3* and *EphB1*. For the B-class receptors *EphB2*, *EphB3* (6/8 osteosarcoma samples), *EphB4* and *EphB6* (5/8 osteosarcoma samples) an expression level above background in osteosarcomas, but no differential expression was detected. With the exception of *EphB4*, ephrinB receptor expression in HOBC was below background.

Ephrin proteins are expressed at elevated levels in osteosarcomas

To confirm the differential expression of ephrins at the protein level, we stained osteosarcoma tissue samples (n=10 primary tumors and n=7 metastases) using anti-*EFNA1*, anti-*EFNA2*, anti-*EFNB1*, anti-*EFNB3* and anti-*EphA2*-specific antibodies. For *EFNA1*, *EFNB1* and *EphA2* a tissue microarray harboring several non-neoplastic human bone samples (n=18) were stained in addition. The immunoreactive scores for all antigens are shown in Tab. 4.

EFNA1 protein levels are increased in osteosarcomas

As already suggested from the microarray analysis, a significant upregulation of *EFNA1* was detected in osteosarcomas compared to non-neoplastic bone (p=0.0003). *EFNA1* expression was observed in 7 out of 8 cases of both fetal and adult osteocytes, in osteoblasts and in osteoclasts of non-neoplastic bone (Fig. 1A), but no significant differences between fetal and normal adult bone were found (p=0.33). However, *EFNA1* was detectable at higher intensities in all osteosarcoma samples, where nearly 80% of tumor cells stained positive (Fig. 1B), yet no significant differences between conventional osteosarcomas and metastases were found (p=1). The *EFNA1* immunoreactivity was localized to the membrane and to the cytoplasm of the tumor cells. Interestingly, five osteosarcoma samples showed a nuclear *EFNA1* expression (Fig. 1C).

EphA2 protein is expressed de novo in osteosarcomas

In contrast to the EFNA1 ligand, the EphA2 receptor was not present in fetal and adult non-neoplastic bone (Fig. 1D), but 13/17 osteosarcoma samples showed expression of the EphA2 receptor (Fig. 1E, F). The EphA2 immunoreactivity was largely confined to tumor cells around the bone formations (Fig. 1E arrow). Again, a significant upregulation of EphA2 in osteosarcomas compared to non-neoplastic bone could be confirmed at the protein level ($p=0.000024$), no significant differences between conventional osteosarcomas and metastases were found ($p=1$).

Differential expression of EFNB1, EFNA2 and EFNB3

4/8 fetal bone samples showed a cytoplasmic expression of EFNB1 (Fig. 2A), while no EFNB1 protein was found in the adult bone (Fig. 2B). The EFNB1 immunoreactivity was largely confined to the osteoblasts of fetal bone and to the tumor cells around the bone formations. In contrast to adult bone, all osteosarcoma tissue samples showed an EFNB1 protein expression (Fig. 2C, D). A significant upregulation of EFNB1 in osteosarcomas compared to non-neoplastic bone could be confirmed at the protein level ($p=0.000054$). Like EphA2 receptor, the EFNB1 ligand appeared *de novo* expressed in osteosarcoma primary tumors, but was significantly downregulated in the metastases as compared to conventional osteosarcomas ($p=0.015$). EFNA2 (Fig. 3A, B) and EFNB3 (Fig. 3C, D) showed no significant differences between conventional osteosarcomas and metastases ($p=0.3$ and $p=1$, respectively). The EFNA2, EFNB1 and EFNB3 immunoreactivity was localized to the cytoplasm of the tumor cells. Interestingly, an expression of EFNA2 and EFNB3 was also found in the endothelial cells of tumor blood vessels (Fig. 2B, D arrows).

EFNA1-mediated EphA2 receptor phosphorylation and internalization

The deregulation of EFNA1 or EphA2 and the EFNA1-EphA2 ligand-receptor signaling plays an important role in tumor development and metastasis in several carcinomas. To investigate

the potential of ephrin signaling in osteosarcomas, we further analyzed EFNA1 and EphA2 expression and regulation *in vitro*. Osteosarcoma cell lines were tested for their EFNA1 and EphA2 protein expression. Using Western blot, different levels of EFNA1 ligand and EphA2 receptor protein could be detected in the cell lines (Fig. 4A). SaOS2 expressed the highest level of EphA2, while only a weak EphA2 expression could be observed in HOBc. EFNA1 was detected at nearly similar levels in all cell lines, with slightly lower levels in ZK58, SaOS2 and HOBc.

EFNA1-mediated aggregation of EphA2 receptors stimulates receptor internalization and degradation in breast and prostate carcinomas^{14, 15}. Because of the high EphA2-expression in SaOS2 and MNNG/HOS, these cell lines were used for further analysis. The EphA2 subcellular localization without addition of the soluble ligand revealed a membranous expression at the sites of SaOS2 cell-cell contact and enrichment within membrane ruffles (Fig. 4B). In MNNG/HOS cells, EphA2 was mainly concentrated at sites of cell-cell contact (Fig. 4C). Analysis of EphA2 localization following the addition of EFNA1/F_c revealed a redistribution of the EphA2 receptor from the plasma membrane within 10min after treatment of SaOS2 and MNNG/HOS cells (Fig. 4B, C). EphA2 immunoreactivity as measured by Western blot decreased within one hour following EphrinA1/F_c exposures and 70-90% of the EphA2-protein was lost after 2h in SaOS2 and MNNG/HOS cells (Fig. 5A, B).

EphA2 is enriched at cell-cell contacts in non-transformed epithelial cells where it binds the ligand and becomes phosphorylated¹⁶. In carcinoma cells, EphA2 often remains non-phosphorylated because overexpression is accompanied by an altered EphA2 localization resulting in a failure to bind the EFNA1 ligand¹⁶. However, unlike other tyrosine receptor kinases, EphA2 receptors have a kinase activity independent of phosphorylation and are even assumed to participate in **prostate** cancer metastasis^{11, 16}. In SaOS2 and MNNG/HOS cells the EphA2 receptor is overexpressed and localized in membrane ruffles and at cell-cell-contacts. We aimed to determine whether EphA2 receptors are potentially phosphorylated in the

presence of the EFNA1/F_c soluble ligand, because we showed that both ligand and receptor are present in osteosarcomas. In both osteosarcoma cell lines, an increase in phospho-tyrosine protein level was detectable 2min after binding of the soluble ligand (Fig. 5A, B). After two hours, only weak phosphorylation could be detected, which was consistent with the internalization of the EphA2 receptor following ligand binding. These results suggested that in osteosarcoma cells the interaction between EphA2 receptor and EFNA1 ligand, both overexpressed in tumor samples, might result in phosphorylation and activation of the EphA2 receptor.

EFNA1-ligand binding to the EphA2 receptor induces activation of MAPK signaling

Previous reports suggested that in breast and prostate carcinoma cells ephrin receptor-ligand interaction results in receptor phosphorylation and downregulation of phospho-Erk1/2 levels through a negative feedback-loop¹⁴. In contrast, *Pratt et al. 2002* found an increased Erk1/2 activity after treatment with EFNA1/F_c in MDA-MB-231 breast cancer cells¹⁵. To determine the effects of EphA receptor activation following ligand application in osteosarcomas, we assessed Ras-MAPK-signaling through Western blot analysis following EFNA1/F_c ligand binding. In SaOS2 and MNNG/HOS cells Erk1/2 phosphorylation increased within 2min after EFNA1 ligand treatment, peaked at 2h and 30min, respectively and then slowly declined (Fig. 5A, B). These data clearly demonstrate that elevated levels of both EFNA1 ligand and EphA2 receptor in osteosarcomas are likely to contribute to an increased mitogenic signaling via the Ras-MAPK pathway.

EFNA1-EphA2 signaling enhances cell migration in osteosarcoma cells

Previous reports have suggested an involvement of the EphA receptor class in breast cancer cell migration and invasion¹⁷. Therefore we tested the effect of EphA2 activation by EFNA1/F_c on anchorage-independent and anchorage-dependent growth and migration of

EphA2-expressing cells. MNNG/HOS cells were treated with 1µg/ml EFNA1/F_c for 96h and cell growth was analyzed. MNNG/HOS cells showed a small increase in growth on plastic 96h after EFNA1/F_c-treatment (Fig. 6A). In addition, MNNG/HOS cells also showed a 20% increase in anchorage-independent growth, suggesting a limited growth stimulatory role of EFNA1-EphA2 signaling (Fig. 6B). For migration studies, MNNG/HOS cells were treated with 1µg/ml EFNA1/F_c up to 48h (Fig. 6C). A slight increase in migration using the soluble ligand compared to untreated cells was observed after 24h and 48h.

EFNA1/F_c mediated stimulation of EphA2 induces autoregulation of EphA2 and EFNA1

Earlier investigations had suggested that EphA2 expression can be stimulated via EGFR and the downstream Mek/Erk signaling in the human epidermal carcinoma cell line A431 and in the head and neck carcinoma cell line HN5¹⁸. Our investigations in osteosarcoma cells now revealed a role for the EphA2 receptor itself in the activation of this pathway. Therefore we investigated whether the *EphA2* and *EFNA1* genes might exert an autoregulation following EFNA1 ligand binding. Treatment of MNNG/HOS cells with the ligand induced *EFNA1* mRNA after 5min and the elevated levels persisted up to 48h (Fig. 7A). *EphA2* mRNA showed a slight upregulation after 1h in MNNG/HOS cells, however overall the induction was weak (Fig. 7A). These results indicate a potential autoregulatory loop, which increases mainly *EFNA1* mRNA levels in osteosarcoma cells upon ligand binding. To define to what extent the EFNA1 and EphA2 autoregulation was MAPK-dependent we treated MNNG/HOS cells with the Mek-inhibitor U0126 and measured protein and mRNA expression of EphA2 and EFNA1 by Western blot and RT-PCR, respectively. Both EphA2 and EFNA1 protein and mRNA were reduced through treatment with U0126 (Fig. 7B).

Discussion

Today, a number of molecular markers have been identified in osteosarcomas, some of which have the potential to act as novel therapeutic targets¹⁹. For example VEGF expression in osteosarcoma correlates with the level of microvascular density and the presence of pulmonary metastases²⁰. In addition, increased levels of MMP9 are associated with increased metastatic potential and the receptor tyrosine kinases ErbB2/Her-2 and c-Met are overexpressed and/or amplified in bone tumors²¹⁻²³. Current treatment procedures involve surgical resection and neoadjuvant chemotherapy involving methotrexate, cisplatin, and adriamycin and have resulted in tumor-type dependent response rates up to 80%. Nevertheless, a significant fraction of patients (app. 30%) die because of metastases. Therefore, an improved understanding of the molecular mechanisms driving osteosarcoma progression and metastasis and the establishment of novel diagnostic and therapeutic targets is urgently needed.

Using microarray analysis, we detected a distinct expression profile for several ephrins and their receptors in human osteosarcoma tissue samples when compared to non-neoplastic human bone. The Ephrin receptors and ligands are associated with tumor development, progression and metastasis in numerous human cancers. The EphA2 receptor is upregulated in colorectal, lung and breast cancer, as well as in melanomas and is often found to correlate with tumor stage²⁴. Several publications described a role in angiogenesis, migration and tumor compartmentalization^{25, 26}. However, a functional diversity was observed within the ephrin family. For example, an oncogenic role was found for the EphA/EFNA ligand-receptor system but a tumor suppressive role for EphB receptors^{27, 28}. In osteosarcoma, ephrin expression was first described by *Varelias et al. (2002)*, who found distinct expression profiles for several ephrins within tumor samples and osteosarcoma cell lines⁶. Using microarray analysis and immunohistochemistry we extended these data and confirmed high overexpression of the EphA2 receptor and the EFNA1 ligand in the majority of human

osteosarcomas. While the ligand was also detected in fetal and adult normal tissue but strongly upregulated in tumor tissue, *de novo* expression was detected for the EphA2 receptor in tumor tissue. This indicates that during osteosarcoma development a potential change in ligand-receptor interaction within the A-class of ephrins might occur. The EFNA1 ligand is able to bind the EphA2 receptor with high affinity²⁹, yet binds also other members of the A-class receptors. Absence of EphA2 from fetal and adult normal human bone, but a strong upregulation during tumor development suggests a specific role for the EFNA1 ligand-EphA2 receptor interaction in osteosarcoma. Our microarray data also imply upregulation of the EphA3 receptor in osteosarcomas. This suggests that EFNA1 might also interact with this receptor, however at the moment an involvement of EphA3 in osteosarcoma is unknown.

In cells derived from breast cancer and other carcinomas, controversial results were also reported with respect to signaling cascade activation upon EFNA1-mediated EphA2 stimulation. *Macrae et al.* found a negative feedback loop involving transcriptional regulation of EphA2 and suppression of Ras function¹⁴, also *Wykosky et al.* recently suggested that soluble EFNA1 upon interaction with EphA2 suppresses Ras/MAPK signaling in cells derived from glioblastoma multiforme³⁰. In contrast, others suggested activation of MAPK signaling through the EFNA1 ligand¹⁵. Stimulation of osteosarcoma cells with the soluble EFNA1 ligand induced MAPK signaling as indicated by Erk1/2 phosphorylation. Activation of MAPK signaling and the control of downstream effectors such as Fos and Jun have key cellular functions such as proliferation and migration and are linked to osteosarcoma development³¹. In contrast to the data described in breast and also in prostate cancer cells, applications of the EFNA1 ligand to MNNG/HOS cells induced migration and to a lower extend also proliferation. This observation indicates that EFNA1 EphA2 upregulation in osteosarcoma might be positively involved in the known oncogenic pathways responsible for osteosarcoma formation and progression. Interestingly our data also suggest a positive feedback loop including the EFNA1 EphA2 ligand-receptor system and acting via

Ras/MAPK. In prostate cancer cells the EFNA1 ligand induces dephosphorylation of focal adhesion kinase (FAK) followed by inhibition of cell spreading and migration³². *Parri et al. 2005* extended these findings and demonstrated a repulsive response of PC3 prostate cancer cells following EFNA1 application; however in the absence of FAK dephosphorylation³³. These observations are in line with our data indicating a migratory response of osteosarcoma cells *in vitro*. We were however unable to detect FAK dephosphorylation following EFNA1 application (data not shown). Thus it remains to be determined to what extent FAK is involved in EFNA1-induced migration of osteosarcoma cells and suggests a totally different role in growth and migration control of EFNA1 ligand-EphA2 receptor interaction in bone tumors as compared to epithelial tumors.

The strong upregulation of EFNA1 ligand and EphA2 receptor in osteosarcomas marks the ephrins as interesting potential therapeutic target. *Noberini et al (2008)* showed, that small molecules are able to selectively inhibit the interaction of EphA2 with its natural ligand³⁴. These compounds also inhibit ephrin-induced phosphorylation of EphA2 without affecting cell viability or the phosphorylation of other receptor tyrosine kinases. Thus, further analysis on the role of EFNA1 and EphA2 in osteosarcomas might determine whether EphA2 is a possible therapeutic target for the treatment with small inhibitory molecules in osteosarcoma patients.

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For Peer Review

Tables

Table 1: Tissue from patients with osteosarcoma used for microarray and immunohistochemical analyses. * used for microarray and immunohistochemical analyses.

+: not used for immunohistochemical staining. f = female. m = male.

Number	Type	Sex	Age	Localization
OS1*	conventional	f	15	Femur
OS2	conventional	m	16	Tibia
OS3	conventional	m	74	Humerus
OS4*	metastasis	m	40	Lung
OS5	metastasis	m	59	Lung
OS6*	metastasis	f	21	Lung
OS7	metastasis	f	20	Lung
OS9*	conventional	m	23	Tibia
OS10	metastasis	m	24	Lung
OS11	metastasis	f	45	Lung
OS12*	metastasis	m	67	Lung
OS14	conventional	m	56	Scapula
OS15*	conventional	f	74	Femur
OS16* ⁺	conventional	f	37	Lung
OS17	conventional	m	54	Tibia
OS18*	conventional	m	7	Femur
OS20	conventional	m	33	Femur
OS21	conventional	m	59	Femur
OS24*	conventional	m	17	Femur

Table 2: Genes with at least 10 fold overexpression (**A**) or suppression (**B**) in osteosarcoma compared to HOBC. Fc: fold change. ID: Affymetrix identity.

A: Genes with at least 10 fold overexpression in osteosarcomas compared to HOBC

ID	Symbol	fc	Gene
207370_at	IBSP	41.7	integrin-binding sialoprotein
201721_s_at	LAPTM5	29.8	lysosomal multispinning membrane protein 5
218678_at	NES	28.7	nestin
217028_at	CXCR4	25.7	chemokine (C-X-C motif) receptor 4
201720_s_at	LAPTM5	23.4	lysosomal multispinning membrane protein 5
204712_at	WIF1	22.2	Wnt inhibitory factor-1
203305_at	F13A1	20.6	coagulation factor XIII, A1 polypeptide
213068_at	DPT	19.6	dermatopontin
215049_x_at	CD163	19.0	CD163 molecule
201117_s_at	CPE	18.6	carboxypeptidase E
204379_s_at	FGFR3	17.8	fibroblast growth factor receptor 3
201116_s_at	CPE	17.5	carboxypeptidase E
202878_s_at	CD93	15.9	Complement component 1, q subcomponent, receptor 1
207977_s_at	DPT	15.8	dermatopontin
215783_s_at	ALPL	14.5	alkaline phosphatase, liver/bone/kidney
217897_at	FXVD6	14.5	FXVD domain containing ion transport regulator 6
206488_s_at	CD36	13.7	CD36 molecule (thrombospondin receptor)
203645_s_at	CD163	13.6	CD163 molecule
221558_s_at	LEF1	13.5	lymphoid enhancer-binding factor 1
218002_s_at	CXCL14	13.4	chemokine (C-X-C motif) ligand 14
202023_at	EFNA1	13.0	ephrin-A1

209087_x_at	MCAM	12.2	melanoma cell adhesion molecule
219478_at	WFDC1	12.1	WAP four-disulfide core domain 1
266_s_at	CD24	12.0	CD24 molecule
202746_at	ITM2A	11.4	integral membrane protein 2A
208146_s_at	CPVL	11.1	carboxypeptidase, vitellogenic-like
219607_s_at	MS4A4A	11.0	membrane-spanning 4-domains, subfamily A, member 4
211343_s_at	COL13A1	10.7	collagen, type XIII, alpha 1
202747_s_at	ITM2A	10.6	integral membrane protein 2A
214574_x_at	LST1	10.2	leukocyte specific transcript 1
209301_at	CA2	10.1	carbonic anhydrase II

B: Genes with at least 10 fold suppression in osteosarcomas compared to HOBc

ID	Symbol	fc	Gene
209395_at	CHI3L1	123.9	chitinase 3-like 1 (cartilage glycoprotein-39)
209396_s_at	CHI3L1	87.0	chitinase 3-like 1 (cartilage glycoprotein-39)
205792_at	WISP2	65.4	WNT1 inducible signaling pathway protein 2
203851_at	IGFBP6	37.6	insulin-like growth factor binding protein 6
218468_s_at	GREM1	31.8	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)
204948_s_at	FST	27.2	follistatin
212143_s_at	IGFBP3	23.3	insulin-like growth factor binding protein 3
206157_at	PTX3	22.6	pentraxin-related gene, rapidly induced by IL-1 beta
201107_s_at	THBS1	21.6	thrombospondin 1
209355_s_at	PPAP2B	20.3	phosphatidic acid phosphatase type 2B
203963_at	CA12	20.0	carbonic anhydrase XII
202949_s_at	FHL2	19.4	four and a half LIM domains 2
204508_s_at	CA12	18.2	carbonic anhydrase XII
202912_at	ADM	18.0	adrenomedullin
209687_at	CXCL12	17.6	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
205475_at	SCRG1	16.6	scrapie responsive protein 1
206172_at	IL13RA2	16.0	interleukin 13 receptor, alpha 2
201108_s_at	THBS1	15.2	thrombospondin 1
213112_s_at	SQSTM1	14.2	sequestosome 1
203939_at	NT5E	13.4	5'-nucleotidase, ecto (CD73)
212226_s_at	PPAP2B	12.9	phosphatidic acid phosphatase type 2B

201109_s_at	THBS1	12.7	thrombospondin 1
212344_at	SULF1	12.6	sulfatase 1
214767_s_at	HSPB6	12.3	heat shock protein, alpha-crystallin-related, B6
205397_x_at	SMAD3	12.2	SMAD family member 3
212230_at	PPAP2B	12.1	phosphatidic acid phosphatase type 2B
202275_at	G6PD	12.0	glucose-6-phosphate dehydrogenase
208502_s_at	PITX1	11.7	paired-like homeodomain 1
221111_at	IL26	11.4	interleukin 26
202434_s_at	CYP1B1	11.3	cytochrome P450, family 1, subfamily B, polypeptide 1
202436_s_at	CYP1B1	11.3	cytochrome P450, family 1, subfamily B, polypeptide 1
212992_at	AHNAK2	11.2	AHNAK nucleoprotein 2
204037_at	LPAR1	11.0	lysophosphatidic acid receptor 1
207345_at	FST	10.9	follistatin
202765_s_at	FBN1	10.5	fibrillin 1
204036_at	LPAR1	10.4	lysophosphatidic acid receptor 1
205924_at	RAB3B	10.3	RAB3B, member RAS oncogene family

Table 3: Ephrin expression profile in osteosarcoma tissue samples compared to the non neoplastic osteoblast primary culture HOBc. Relative expression levels below 1,000 were defined as background. OS: osteosarcoma.

Relative Expression	A-Ligand	A-Receptor	B-Ligand	B-Receptor
significant (>1,000)	EFNA1 (HOBc below 1,000, 8/8 OS above 1,000) EFNA4 (1/8 OS above 1,000, HOBc below 1,000)	EphA1 (1/8 OS above 1000, HOBc below 1,000) EphA2 (all probes above 1,000) EphA3 (HOBc and 1/8 OS below 1,000) EphA4 (1/8 OS above 1,000, HOBc below 1,000) EphA5 (1/8 OS above 1000, HOBc below 1,000)	EFNB2 (all probes above 1,000)	EphB2 (HOBc below 1,000, 8/8 OS above 1,000) EphB3 (HOBc and 2/8 OS below 1000) EphB4 (all probes above 1,000) EphB6 (5/8 OS above 1,000)
not significant (<1,000)	EFNA2 (all probes below 1,000) EFNA3 (all probes below 1,000) EFNA5 (all probes below 1,000)	EphA7 (all probes below 1,000)	EFNB1 (all probes below 1,000) EFNB3 (all probes below 1,000)	EphB1 (all probes below 1,000)

Table 4: Immunohistochemical analyses of EFNA1, EFNA2, EFNB1, EFNB3 and EphA2 in human osteosarcoma and non-neoplastic bone tissue samples. The immunohistochemical staining was calculated through the immunoreactive score (IRS) of the whole tissue sample. PP: percentage of positively stained cells. SI: intensity of positively stained cells.

		Osteosarcoma			Bone		
		Conventional	Metastases	Total	Fetal	Adult	Total
EFNA1 ligand	SI	2.6	2.6	2.6	0.9	1.9	1.5
	PP	3.8	3.3	3.6	1.3	2.4	1.9
	IRS	10.1	9.1	9.7	2.6	4.7	3.8
EFNA2 ligand	SI	2.1	2.1	2.1			
	PP	3.3	2.9	3.1			
	IRS	7.9	6.1	7.2			
EFNB1 ligand	SI	2.7	1.9	2.4	1.6	0.0	0.7
	PP	3.0	2.1	2.6	1.0	0.0	0.4
	IRS	8.1	4.3	6.5	3.3	0.0	1.4
EFNB3 ligand	SI	2.0	2.0	2.0			
	PP	3.0	2.4	2.8			
	IRS	6.9	5.7	6.4			
EphA2 receptor	SI	1.0	1.3	1.1	0.0	0.0	0.0
	PP	1.8	1.3	1.6	0.0	0.0	0.0
	IRS	2.4	2.6	2.5	0.0	0.0	0.0

Figure legends

Figure 1: Expression of EFNA1 ligand and EphA2 receptor in non-neoplastic human bone and in osteosarcoma tissue samples. (A) EFNA1 expression in osteocytes (red arrow), osteoblasts (black arrow) and osteoclasts (blue arrow) of non-neoplastic bone. (B) EFNA1 expression in osteosarcoma. (C) Strong nuclear expression of EFNA1 in osteosarcoma cells. (D) EphA2 is not expressed in non-neoplastic bone. (E) EphA2 staining was predominantly observed in tumor cells around neoplastic bone formations (black arrow). (F) Weak to moderate cytoplasmic expression of EphA2 in high grade osteosarcoma. LSAB-HRP staining was used.

Figure 2: Expression of EFNB1 ligand in non-neoplastic human bone and in osteosarcoma tissue samples. (A) EFNB1 expression in fetal non-neoplastic bone. (B) EFNB1 is not expressed in adult non-neoplastic bone. (C) EFNB1 expression in conventional osteosarcoma. (D) EFNB1 expression in osteosarcoma metastases. LSAB-HRP staining was used.

Figure 3: Expression of EFNA2 ligand and EFNB3 ligand in conventional and osteosarcoma metastases. (A) EFNA2 expression in conventional osteosarcoma. (B) EFNA2 expression in tumor vessels (arrow). (C) EFNB3 expression in conventional osteosarcoma. (D) EFNB3 expression in osteosarcoma metastases and tumor vessels (arrow). LSAB-HRP staining was used.

Figure 4: Internalization of EphA2 receptor following EFNA1 ligand application. (A) *left panel*: protein expression of EFNA1 ligand and EphA2 receptor in primary osteoblast cells and in osteosarcoma cell lines. Actin levels served as a loading control. 1: ZK58. 2: MG63. 3: MNNG/HOS. 4: SaOS2. 5: SJSA. 6: OST. 7: HOS. 8: HOBc. *Right panel*: Semi-quantitative

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evaluation of EphA2 receptor and EFNA1 ligand levels. **(B, C)** Time-dependent internalization of EphA2 receptor following EFNA1/F_c application in SaOS2 and MNNG/HOS cells. EphA2-protein was accumulated in membrane ruffles of SaOS2 (B arrow) and on sites of cell-cell contact in MNNG/HOS cells (C arrow). Nuclei were counterstained with DAPI. Magnification 1,000x.

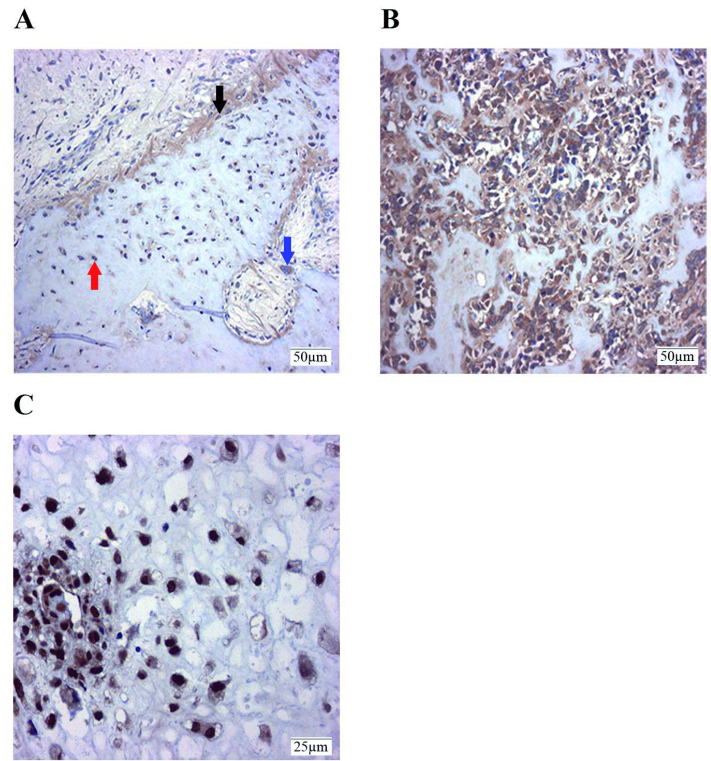
Figure 5: Activation of P-Erk1/2 via EFNA1-stimulated receptor activation. SaOS2 (A) and MNNG/HOS (B) cells were stimulated with 1µg/ml EFNA1/F_c for the indicated time points. Total cell lysates were blotted for phospho-tyrosine (PY). The membranes were then stripped and reprobed for EphA2, P-Erk1/2 and Erk1. *Lower panel of A and B:* For semi-quantification of Western blot results, ImageJ was used. ut: untreated cells.

Figure 6: Receptor stimulation via EFNA1 ligand has a limited effect onto cell growth. MNNG/HOS cells were plated on 96-well-plates or polyhema plates and treated with 1µg/ml EFNA1/F_c. Anchorage-dependent **(A)** and anchorage-independent **(B)** proliferation of cells was measured using a MTT assay and the mean absorbance is shown. As a control, untreated cells were used. MNNG/HOS cells showed slightly increased anchorage-independent proliferation when treated with the soluble ligand. All assays were performed as triplicates and repeated at least 2-times and one representative example is shown. **(C)** Wound-healing assays of exponentially growing MNNG/HOS cells. Confluent cells were wounded through the central axis following treatment with 1µg/ml EFNA1/F_c or PBS as a control up to 48h. Migration was analyzed using a phase contrast microscope (Leica). Magnification 200x. All experiments were repeated at least 2-times and one representative example is shown.

Figure 7: Autoregulation of EFNA1 ligand and EphA2 receptor through EFNA1/F_c treatment and Mek activation. (A) MNNG/HOS cells were treated with 1µg/ml EFNA1/F_c

for the time points indicated. RNA were isolated and analyzed via RT-PCR for *EFNA1 ligand* and for *EphA2 receptor* expression. *Actin* levels served as loading control. For semi-quantification of RT-PCR results, ImageJ was used. ut: untreated cells. **(B)** MNNG/HOS cells were treated with 50 μ M U0126 for 48h (+). As a control, cells were treated with the solvent DMSO (-). *left panel*: Cells were harvested and equal amounts of cell lysates were resolved by SDS-PAGE and analyzed through Western blot for EphA2, EFNA1 and P-Erk1/2 expression. Actin levels served as loading control. *right panel*: RNA was extracted following RT-PCR reaction for *EphA2* and *EFNA1* expression. Amplification of *Actin* as loading control was performed. ut: untreated cells. For semi-quantification of RT-PCR and Western blot results, ImageJ was used. All experiments were repeated at least 2-times and one representative example is shown.

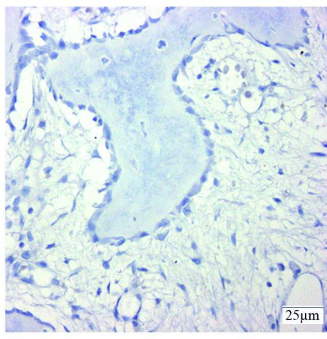
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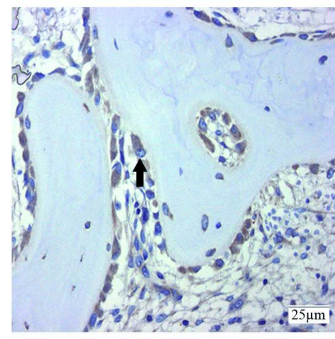
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Figure 1

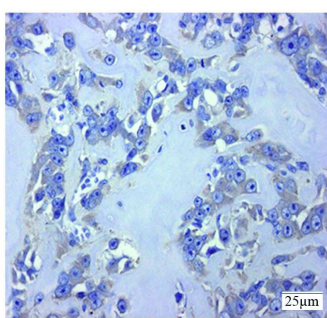
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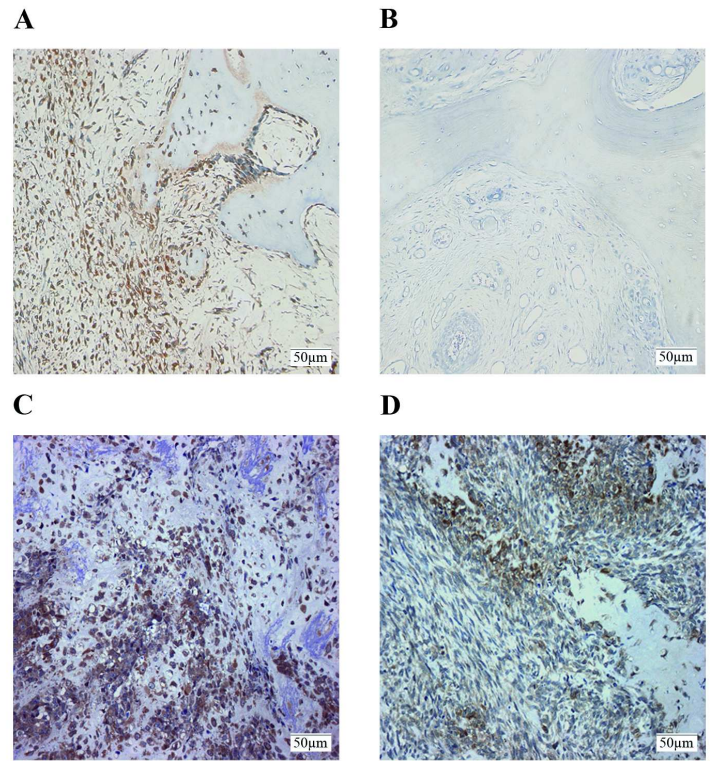


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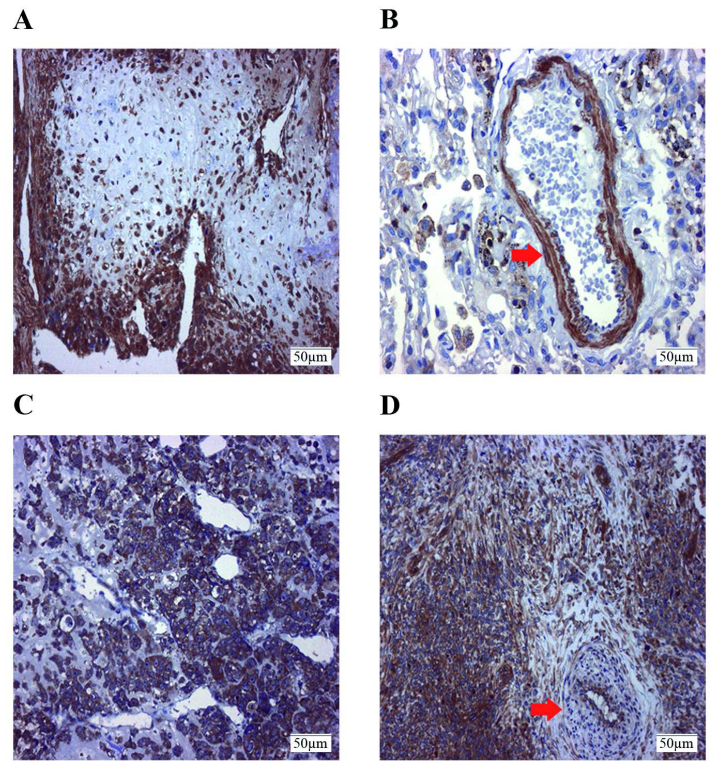
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Figure 2



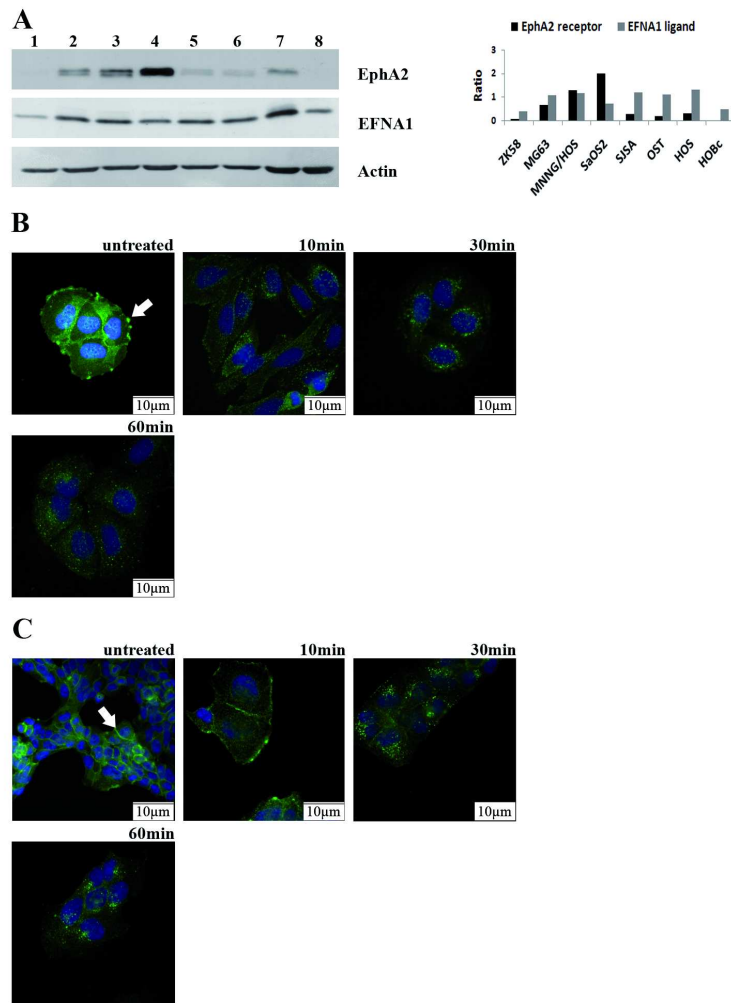
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Figure 3



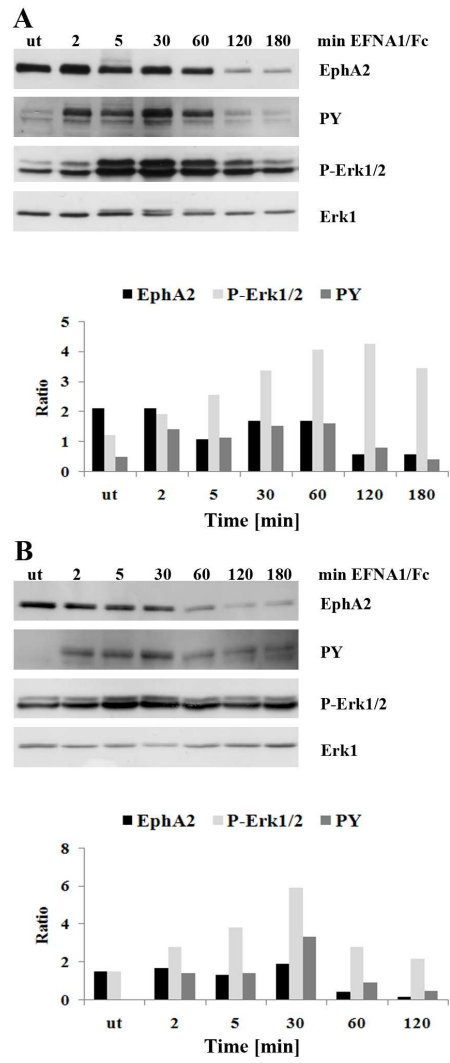
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Figure 4



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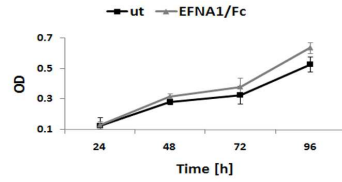
Figure 5



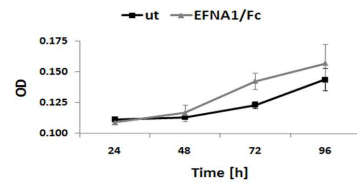
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Figure 6

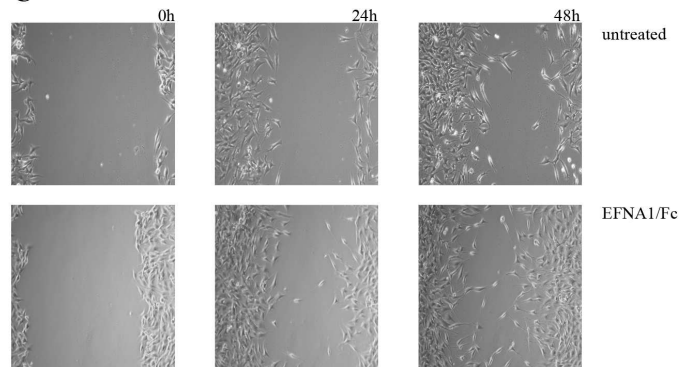
A



B

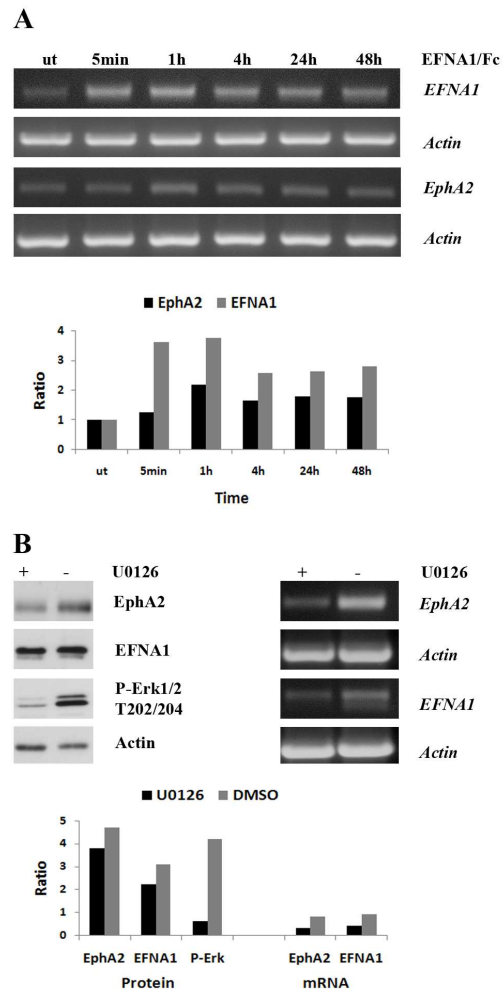


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Figure 7



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