

## Microcracks and Osteoclast Resorption Activity In Vitro

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**Abstract** During bone remodeling osteoclasts resorb bone, thus removing material, e.g., damaged by microcracks, which arises as a result of physiological loading and could reduce bone strength. Such a process needs targeted bone resorption exactly at damaged sites. Osteocytic signaling plays a key role in this process, but it is not excluded that osteoclasts per se may possess toposensitivity to recognize and resorb damaged bone since it has been shown that resorption spaces are associated with microcracks. To address this question, we used an in vitro setup of a pure osteoclast culture and mineralized substrates with artificially introduced microcracks and microscratches. Histomorphometric analyses and statistical evaluation clearly showed that these defects had no effect on osteoclast resorption behavior. Osteoclasts did not resorb along microcracks, even when resorption started right beside these damages. Furthermore, quantification of resorption on three different mineralized substrates, cortical bone, bleached bone (bone after partial removal of the organic matrix), and dentin, revealed lowest resorption on bone, significantly higher resorption on bleached bone, and highest resorption on dentin. The

difference between native and bleached bone may be interpreted as an inhibitory impact of the organic matrix. However, the collagen-based matrix could not be the responsible part as resorption was highest on dentin, which contains collagen. It seems that osteocytic proteins, stored in bone but not present in dentin, affect osteoclastic action. This demonstrates that osteoclasts per se do not possess a toposensitivity to remove microcracks but may be influenced by components of the organic bone matrix.

**Keywords** Osteoclast · Microcrack · Mineralized tissue · Toposensitivity

Bone is a living tissue which is continuously renewed through the process of remodeling. This remodeling is crucial for maintaining skeletal structure and function and the adaptation of the skeleton to specific mechanical needs [1]. Even normal physical activities may cause microcracks in the skeleton, which then could reduce bone strength [2–8]. Therefore, it is in the interest of architectural stability to remove cracks and, in so doing, to achieve two benefits: first, microcracks cannot increase the risk of fracture due to their accumulation and, second, it is speculated that the removal of microcracks is an essential function of bone turnover [9–11]. Thus, microcracks in bone may be “hot spots” for targeted bone resorption by osteoclasts. Gefen and Neulander [12] described, by computational experiments, that microcracks with a minimal length of 48  $\mu\text{m}$  exhibit the potential to initiate a remodeling process. This may happen via mechanical signals, tissue geometry, or biochemical coupling by cytokines secreted by various cell types in bone [13]. However, no single mechanism has been shown to dominate, but a role in the process of damage repair has been shown for osteocytes. Osteocytes surrounding fatigue

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microcracks *in vivo* undergo apoptosis, which precede the onset of osteoclastic resorption and colocalize with areas of resorbed bone [14]. Furthermore, it is hypothesized that osteoclasts fulfill a phagocytic role in bone guided by the rise of apoptotic osteocytes. The tight spatial and temporal coupling of osteocyte apoptosis and bone damage suggests that osteocyte apoptosis is a key controlling step in targeted osteoclastic resorption [15–17]. Beyond that mechanism, osteoclasts may also be activated via geometric coupling due to changes in tissue architecture. The fact that cells react to topographical and architectural situations is known, e.g., from osteoblasts, which strongly respond to the local geometric environment [18, 19].

Several *in vivo* studies have addressed the relationship between microcracks and osteoclastic resorption activity [20]. Bentolila et al. [21] and Herman et al. [11] showed explicitly that linear microcracks with widths of 1.5–5  $\mu\text{m}$  and lengths of  $\sim 200 \mu\text{m}$  in cortical bone are associated with resorption spaces. Burr and coworkers [10, 22, 23] described in a dog model (by applying loading to limbs at different time points before death) that microcracks are associated significantly more often with resorption spaces than would be expected by random remodeling processes alone. By this they proved a significant increase in remodeling sites occurring subsequent to microdamage initiation. Unfortunately, it is difficult to study the relationship between microcracks and (boosted) remodeling *in vivo* since the number of events of this type is relatively low in any histological slide. Furthermore, it is not really possible to verify the chronological appearance of cracks and lacunae generated during *in vivo* bending experiments. In addition, the result of *in vivo* experiments is always a sum of numerous cell events and does not easily allow differentiation of the specific functions of the particular cell types [24]. This raises the question of whether targeted bone remodeling is due to (1) a direct sensitivity of osteoclasts to bone defects; (2) a sensitivity of osteoblasts, which then signal to osteoclasts; or (3) a signaling to osteoclasts from the osteocytic network.

For this reason, we undertook an *in vitro* study culturing primary osteoclasts only so that possibilities (2) and (3) can be excluded. Hence, our aim was to answer the question whether osteoclasts per se possess toposensitivity and have the intention to remove microcracks by targeted resorption based on this ability.

## Materials and Methods

### Osteoclast Cultures

Osteoclast precursor cells were generated from human peripheral blood mononuclear cells obtained from healthy

donors, as previously described [25, 26]. The experiments were approved by the Austrian Ethics Committee; informed consent was obtained from all volunteers. Blood from eight different donors was analyzed. Briefly, peripheral blood mononuclear cells were isolated by centrifugation over a Lymphoprep gradient and cultured in  $\alpha\text{MEM}$  (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Thermo Fisher, Geel, Belgium), 2 mM L-glutamine, 20 ng/ml macrophage-colony stimulating factor (M-CSF; R&D Systems, Wiesbaden-Nordenstadt, Germany), and 30  $\mu\text{g/ml}$  gentamycin. After 8–10 days in culture, adherent cells were removed using trypsin, seeded at a density of  $6 \times 10^4$  cells/well into 48-well plates containing bone or dentin slices, and cultured in  $\alpha\text{MEM}$  supplemented with 10% FCS, 2 mM L-glutamine, 30  $\mu\text{g/ml}$  gentamycin, 20 ng/ml M-CSF, and 2 ng/ml receptor activator of NF- $\kappa\text{B}$  ligand (RANKL). After 14 days (dentin) or 28 days (bone), slices were removed and prepared for histomorphometric measurements, fixed for immunofluorescence studies, or used for RNA isolation.

For migration experiments, osteoclast precursors were seeded onto one-half of dentin slices, and the other half was covered by silicone. Twenty-four hours later the silicone covering was removed, nonadherent cells were washed away, and incubation was continued by covering the whole slice with medium for 14 days. During that time preosteoclasts fuse and mature osteoclasts arise.

### Preparation of Tissue Substrates

We chose three different mineralized substrates widely used in the literature [27]: bone (bovine cortical bone), bleached bone, and dentin (elephant ivory). Dentin of elephant tusk (ivory) was kindly provided by German Customs in accordance with international laws. Cortical bone parts were removed from the mid-diaphyseal region of femora, cleaned from soft tissue, and stored at  $-25^\circ\text{C}$  until cutting (devitalized bone). Slices of approximately 300  $\mu\text{m}$  thickness were obtained from bone and dentin material by cutting with a diamond saw, followed by polishing with tissue loaded with diamond grains.

For macroscratch, microscratch, and microcrack experiments, slices were polished completely. For migration experiments, slices were half-polished because different surface roughnesses allowed visualization of a defined edge. This was necessary because one-half of those slices were covered by silicone before seeding.

Macroscratches (with widths of 15–20  $\mu\text{m}$ ) were introduced along the complete surface using a scalpel blade. These macroscratches ran in parallel with a distance of 300–500  $\mu\text{m}$ .

Microscratches (very fine, superficial defects with widths of  $\geq 1 \mu\text{m}$  and lengths of a few hundred

micrometers) were introduced onto the surface by scratching over the edge of a glass slide.

Sharp microcracks (with lengths up to 700  $\mu\text{m}$ , widths of about 3  $\mu\text{m}$ ) were introduced by using the indentation-fracture method [28]. In our case, a Vickers microindenter (Anton Paar GmbH, Graz, Austria) with a load up to 2 N was used to produce long, sharp microcracks in a three-point-bending setup.

Deproteinization of bone samples (“bleaching”) was carried out according to Broz et al. [29]. Briefly, bone slices were incubated with 5.5% aqueous sodium hypochlorite (NaOCl) for 3 days at room temperature and replaced with fresh solution every day. Afterward, specimens were rinsed in distilled water for approximately 3 days, with fresh solution three times every day.

### Immunofluorescence

Human osteoclasts were cultured on dentin slices and fixed with 4% paraformaldehyde for 20 min after 14 days. Cells were then permeabilized with 0.1% Triton X-100 for 5 min, blocked in 10% FCS/PBS for 30 min, and incubated with primary vitronectin receptor (VNR) antibody for 60 min. Anti-VNR (CD51/CD61; SeroTec, Dusseldorf, Germany) was used at 1:1,000. Cells were washed three times in PBS and then incubated with Alexa-conjugated secondary antibody at 1:250 (Invitrogen, Life Technologies, Carlsbad, CA). Images were obtained using a Nikon (Dusseldorf, Germany) Eclipse 80i Fluorescence microscope.

### Quantitative Real-Time PCR

Gene expression in osteoclast cultures was assessed by real-time PCR. Total RNA was isolated from preosteoclast cultures and from cultures grown for 14 days on dentin slices (mature osteoclasts) using TRIzol reagent (Invitrogen, Life Technologies). RNA (1  $\mu\text{g}$ ) was reverse-transcribed using the M-MLV Reverse Transcription Kit (Invitrogen, Life Technologies). Quantitative real-time PCR was performed with FastStart SYBR Green Master Mix (Roche, Indianapolis, IN) and selected primers (see below). We used 18S rRNA (TaqMan probe; Applied Biosystems, Foster City, CA) was used as a housekeeping gene for normalization. All PCRs were performed according to the following cycling program: 10 minutes of initial denaturation at 95°C, followed by 60 cycles of 10-s denaturation at 95°C, 45-s annealing at 60°C, and 30-s extension at 72°C. Expression was quantified using the comparative quantification method [30].

Primer sequences were as follows: tartrate-resistant acid phosphatase (TRAP) (forward) 5'-GATCCTGGGTGCA GACTTCA, (reverse) 5'-GCGCTTGAGATCTTAGA GT; cathepsin K (Cath.K) (forward) 5'-TGAGGCTTCT

CTTGGTGTCCATAC-3', (reverse) 5'-AAAGGGTGTCA TTACTGCGGG; calcitonin receptor (CTR) (forward) 5'-GACAAGTGGCTGGCTGAGTG-3', (reverse) 5'-GAAG CAGTAGATGGTCGCAA-3'.

Statistical analysis was done by ANOVA (Prism 4.0; GraphPad Software, San Diego, CA), and data are represented as means  $\pm$  standard deviation (SD).

### Histomorphometric Measurements

Preosteoclasts were seeded onto bone, bleached bone, and dentin slices and kept in culture for 28 days for both bone samples and for 14 days for dentin. Afterward, slices with cells were put into water, sonicated for 10 min to remove cells, and air-dried. Pictures were obtained by reflection light microscopy (objective  $\times$  20) from the entire surface and analyzed with standard image analysis software (ImageJ, [rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/)) [31]. For quantitative analysis, pit areas were defined by an area of resorption surrounded by a margin of unresorbed material. Statistical analysis was done by *t*-test (Prism 4.0), and data are represented as means  $\pm$  SD.

### Calculations of the Probability to Lie on a Microcrack/Scratch

For each donor, pictures were taken from the whole surface of the mineralized substrates as described above. The number of pictures ranged from 9 to 16. In each picture we analyzed what percentage of the resorbed area was lying on the microcrack/scratch.

Based on the measured mean pit area (total pit area divided by pit number), the theoretical probability for a pit touching a microcrack or scratch was calculated according to the following formula (which assumes that the osteoclasts resorb on completely random positions):

$$P = \frac{L(b + 2\sqrt{A/\pi})}{H * B}$$

where *P* is the probability (a number between 0 and 100%), *L* is the length of the microcrack/scratch, *b* is the width of the microcrack/scratch, *A* is the mean pit area ( $\mu\text{m}^2$ ) roughly approximated as a circle, and *H \* B* is the size of the picture.

The theoretical values were then compared with experimentally measured ones. Data are expressed as means  $\pm$  standard error, and statistical analyses were performed using a paired *t* test.

## Results

### Osteoclast Resorption Behavior on Mineralized Tissue

Preosteoclasts were isolated from human peripheral blood mononuclear cells and seeded onto three mineralized

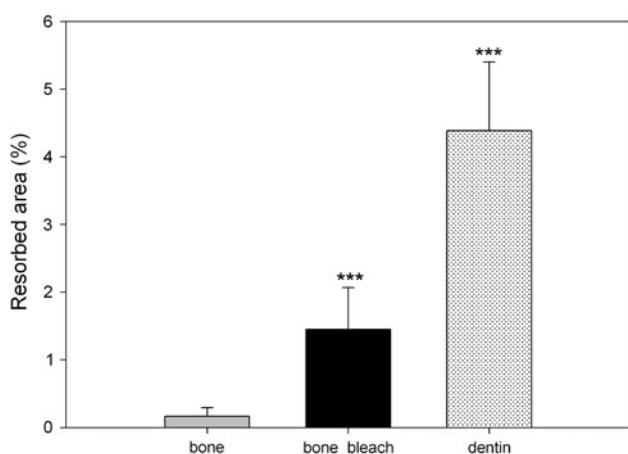
materials exhibiting different characteristic features. The resorption activity of the osteoclasts was evaluated and revealed highly significant differences concerning the resorbed areas. On (devitalized) cortical bone slices, the osteoclasts resorbed 0.16% of the surface, whereas resorption on bleached bone samples accounted for 1.45% and osteoclasts seeded on dentin slices resorbed approximately 4.4% of the surface (Fig. 1).

### Development of Mature Osteoclasts on Mineralized Tissue

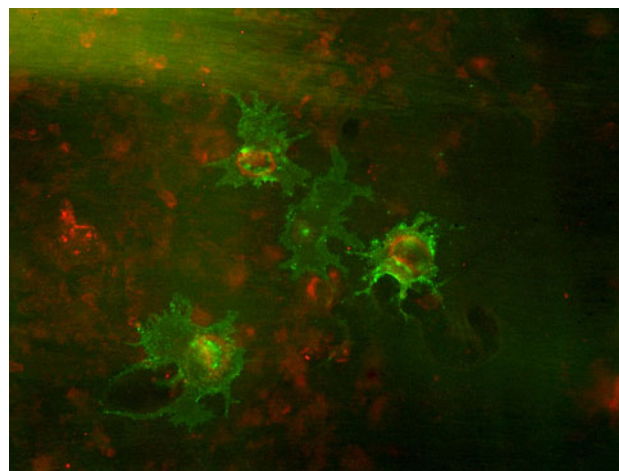
The differentiation of preosteoclasts to mature osteoclasts was confirmed by cell culture on dentin slices, where cells show the characteristic expression of VNR and the typical actin belt formation within a cell, which represents the resorbing organelle during the resorption process itself (Fig. 2). In addition to VNR expression, cells cultured on dentin exhibited very high expression levels of markers characterizing mature osteoclasts, Cath.K, TRAP, and CTR compared to preosteoclastic cells (Fig. 3).

### Assessment of Osteoclastic Resorption

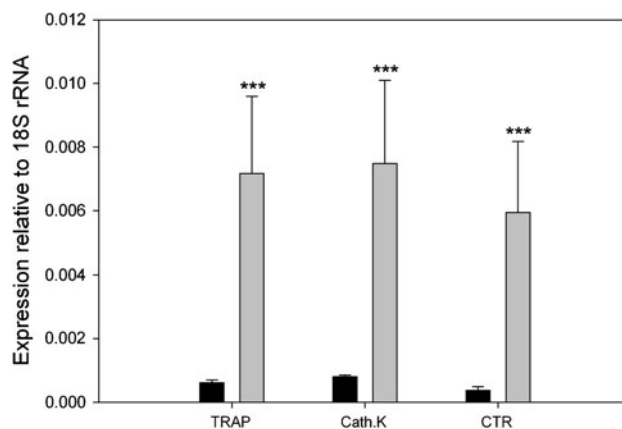
Furthermore, these mature osteoclasts were able to resorb mineralized matrix, as demonstrated by pit formation. Based on this fact, we asked the question, “Do osteoclasts recognize a potential resorbable matrix at distances far away, e.g., in the millimeter range?” The possibility of moving over longer distances would be beneficial for targeted resorption of neighboring “disturbances.” In general, osteoclasts resorb on rough as well as polished surfaces (data not shown). Surprisingly, verification of the



**Fig. 1** Osteoclastic resorption activity on three different mineralized substrates: bone, bleached bone, and dentin. Bars represent mean ± SD; n = 6 different donors, from each of whom resorption was analyzed in parallel on bone, bleached bone, and dentin samples. \*\*\*P < 0.001 (bleached bone vs. bone or dentin vs. bone)



**Fig. 2** Immunofluorescence staining of osteoclast cultures on dentin slices for vitronectin receptor (green) and actin (red)

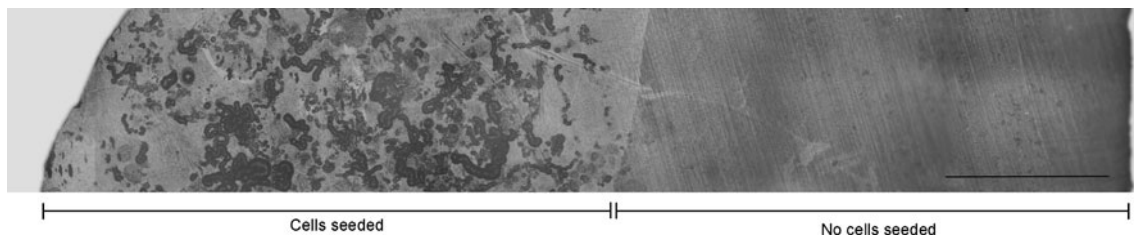


**Fig. 3** TRAP, Cath.K, and CTR mRNA expression of preosteoclasts (black bars) and mature osteoclast cultures (gray bars). The specific gene expression was normalized to 18S rRNA expression. Bars represent mean ± SD (n = 3). \*\*\*P < 0.001 (mature osteoclasts vs. preosteoclasts)

resorption activity showed that resorption of the mineralized surface happened only on the side where cells were seeded (Fig. 4). Mature osteoclasts did not move to the other side of the dentin slice and resorbed only at the place of initial seeding, and even the motivation to meet a resorbable matrix could not encourage cells to move beyond the immediate vicinity. The resorption process itself, which is characterized by pit and trail formation, did not show any preferred orientation and happened within the micrometer range of the cell.

### How Do Osteoclasts Deal with Topographical Discontinuity?

In a first approach to investigate the reaction of osteoclasts to damage in tissue structure, we introduced macroscratches onto the surface of polished dentin slices as a model of



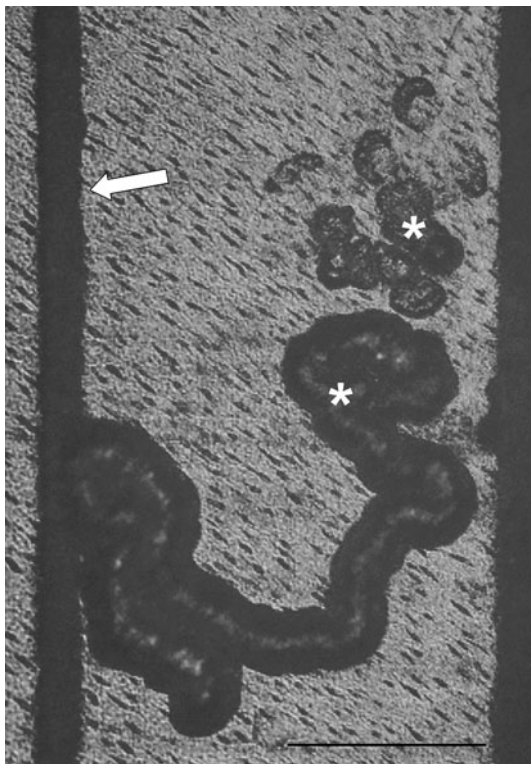
**Fig. 4** Osteoclasts resorbed at the place of initial seeding and do not explore/migrate to neighborhood (millimeters of distance) areas. Cells were seeded onto the left half of the dentin slice; after 14 days,

resorption pit formation could be observed only on that side, but none happened on the other side. Shown is one representative experiment out of five. Scale bar = 1,000  $\mu\text{m}$

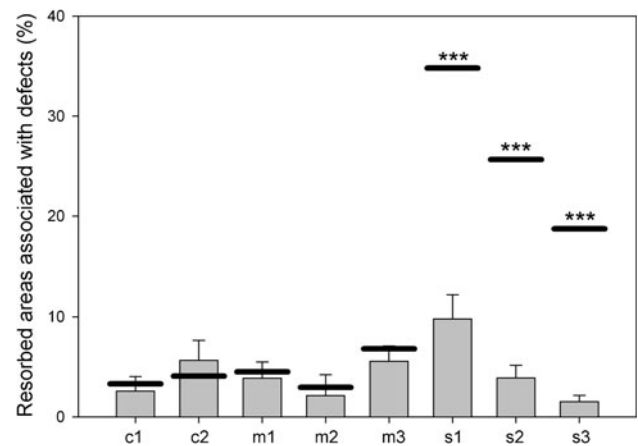
discontinuity in surface topography in mineralized tissue. Qualitative analyses revealed that osteoclasts sense these macroscratches and react to them by changing their resorption direction. In general, osteoclastic resorption took place mainly in between the present macroscratches and showed a clear tendency to be deflected on their edges. Figure 5 shows a typical deflection of osteoclastic resorption progression due to the presence of macroscratches. Only a very low amount of resorption was also performed directly on the macroscratches. Quantitative analyses of this resorption behavior were done by calculating the theoretical probability for resorption pits to lie on a

macroscratch (assuming that there is no preferential resorption anywhere) and to compare it to the experimentally measured values. For all three donors, there was a highly significant lower probability of finding osteoclastic pits on macroscratches in comparison to the theoretical probability of a random (independent) distribution of pits on the surface. For donor 1 (s1), 29% of resorbed areas should be found directly on macroscratches but, in reality, only 8.2% of the pits were verified. For donor 2 (s2), theoretically 25.7% but, in reality, only 3.9% of the resorption pits were discovered on macroscratches. For donor 3 (s3), 18.8% should be positioned directly on macroscratches, but we found just 1.5% on them (Fig. 6), which shows that osteoclasts are clearly deflected by these discontinuities.

Furthermore, we introduced microscratches on osteoclasts. Qualitative analyses showed that osteoclastic resorption happened near such introduced damages and even directly on them. But when resorption started directly on microscratch islands, it ended after the formation of



**Fig. 5** Osteoclasts recognize macroscratches and change their resorption direction. Osteoclasts cultured on dentin containing introduced artificial macroscratches (white arrows). Shown is a typical pattern of resorption pits without scratch contact and of deflected pits due to introduced artefacts (white asterisks). Scale bar = 100  $\mu\text{m}$



**Fig. 6** Theoretical and measured probability for osteoclasts to hit an introduced damage. Plot shows the calculated (black line above the appropriate bar) and measured (gray bar) probabilities of resorption pits laying on an artificially introduced defect. c1, c2, Results for microcracks; m1, m2, m3, results for microscratches; s1, s2, s3, results for macroscratches. Each experiment derives from one donor. Gray bars represent measured values  $\pm$  SE. \*\*\* $P > 0.001$  (calculated vs. measured values for each samples)

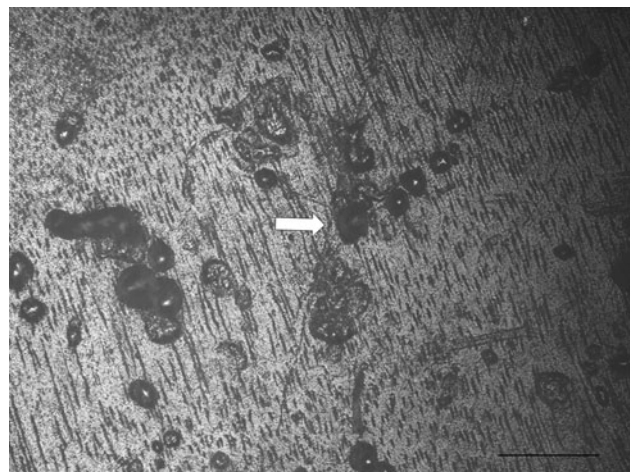
single pits, with no tendency in pit formation progression on the island, which would be absolutely necessary for its removal (Fig. 7). Even more, when osteoclasts passed microscratches during an active resorption process, the progression direction was not changed or deflected due to the presence of such scratches. For quantitative analyses the same calculation as used in macroscratch analyses was applied to this situation and revealed no statistically significant difference between the calculated and measured values. In detail, quantitative analysis for donor 4 (m1) gave a theoretical probability of 4.5% for the resorption pits lying on scratches, whereas the measured value was 3.9%. For donors 5 (m2) and 6 (m3), the calculated rates for pits to lie on microscratches were 2.9 and 6.8%, whereas the real values were found at 2.1 and 5.6%, respectively (Fig. 6).

Macroscratches and microscratches were used to generally investigate the osteoclastic reaction to topographical situations.

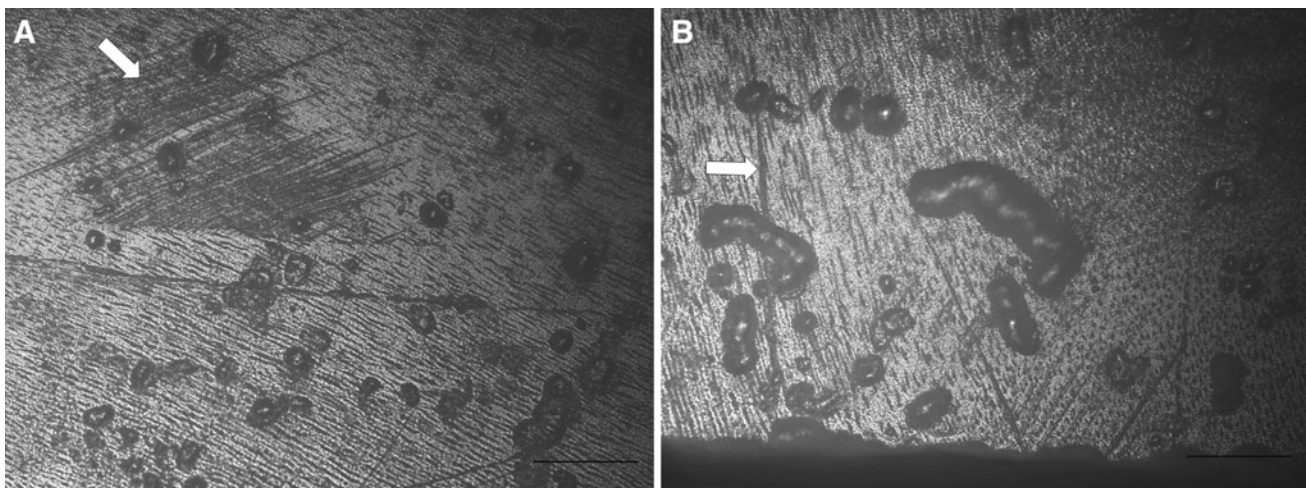
#### How Do Osteoclasts Deal with Microcracks?

To continue the investigation of whether there exists a kind of toposensitivity in osteoclasts, we addressed the more significant question *in vivo*, i.e., the main question of this project, “Do osteoclasts, on their own and without interaction with other cell types, have the potential to sense microcracks and to remove/resorb them in mineralized tissue?”, since it is suggested that targeted bone remodeling (which must be preceded by a targeted resorption process) is initiated by microcracks. To address this issue, microcracks were introduced into dentin slices and introduced to preosteoclasts. Qualitative examination showed that osteoclastic resorption was randomly distributed

throughout the substrate and even started directly on microcracks themselves. But there was no directed resorption along the microcracks, which would be an absolute prerequisite for their removal. Thus, we did not observe any hint of a targeted elimination of these damages in the tissue architecture due to resorption progression (Fig. 8). Quantitative analyses using the same calculations as used for microscratches revealed a theoretical probability for resorption pits to lie on microcracks of 3.3% for donor 7 (c1) compared to a real value of 2.6%. For donor 8 (c2), the calculated probability was 4.1%, whereas in reality 5.7% of the resorption pits were found on microcracks (Fig. 6). In both cases there was no statistically significant difference between the theoretical calculations and the experimental values.



**Fig. 8** Osteoclast resorption activity in correlation to microcrack position (*white arrow*). Osteoclasts by themselves behave in a stochastic manner and do not preferentially resorb microcracks. *Scale bar = 100 μm*



**Fig. 7** Osteoclast resorption behavior is not influenced by the presence of microscratches. Osteoclast resorption activity on dentin surface containing fine superficial scratches. **a** An island of very fine

scratches (*white arrow*). **b** Single fine scratches and passing resorption trails (*white arrows*). *Scale bar = 100 μm*

## Discussion

It was the aim of this project to determine whether osteoclasts possess toposensitivity to recognize microcracks in tissue architecture just by their physical presence.

To choose the optimal substrate, we introduced three different mineralized tissues to osteoclasts: bone, bleached bone, and dentin. Quantitative resorption analyses on flat surfaces of these three mineralized materials revealed lowest resorption on bone, significantly higher resorption on bleached bone (nine times), and highest resorption on dentin (27 times higher than on bone). The difference between bleached and untreated bone may be interpreted as an inhibitory impact of the bone organic matrix. However, the collagen-based matrix itself is not likely to be responsible since resorption is highest on dentin, which consists of collagen and mineral but lacks osteocytic proteins. Hence, we hypothesize that proteins secreted by osteocytes (not present in dentin) and stored in bone matrix could play a role in the inhibition of osteoclast action. These observations are supported by the literature, where it is suggested that (targeted) bone remodeling is controlled through signals from osteocytes [15, 32, 33]. Living osteocytes are able to prevent bone resorption, and damage of the osteocytic canalicular connections is suggested to be the main stimulus to initiate remodeling because large numbers of atypical osteocytes and increased osteocyte apoptosis were found in association with fatigue cracks [14, 21]. In contrast, blocking of osteocyte apoptosis resulted in blocked osteoclastic resorption [15]. In case of apoptosis, factors may be released, which then abrogate the effect of the present proteins from osteocytes [34]. Furthermore, resorption on vital calvarial slices is extremely low compared to devitalized slices [32].

Moreover, it is well known that in vivo mineralized tissue is coated with osteopontin before resorption occurs [35]. In particular, McKee et al. [36] reported that osteopontin covering of particles from bone fracture is a prerequisite for their resorption. The extremely low resorption on bone in our in vitro experiments may also be due to a missing coating, which would otherwise seal the potential inhibitors in the bone matrix and act as a substrate for osteoclast binding. Therefore, we chose dentin as a resorption model to test for the influence of macroscratches, microscratches, and microcracks because it is very similar to bone in chemical composition and structural organization at the micrometer and nanometer levels [37] but allows good osteoclastic resorption.

Preosteoclasts seeded onto dentin slices undergo fusion to mature osteoclasts, which then express typical marker genes (VNR, Cath.K, CTR, and TRAP). Interestingly, these cells showed no intention for active movement over long distances in the millimeter range. Instead, osteoclast

resorption took place right at that position where fusion of precursor cells occurred and movement was restricted to normal resorption progression in the immediate neighborhood. This suggests that targeted bone resorption requires guidance by controlling the fusion site, rather than the motility of resorbing cells.

Osteoclasts seem to be able to react to large topographical discontinuities, such as mounds accumulated on the side of macroscratches, which they cannot cross. However, introduction of microcracks and microscratches into mineralized tissue did not produce mounds acting as physical barriers but, rather, defects from the smooth surface into the depth. The microcracks in our experiments exhibited dimensions (lengths up to 700  $\mu\text{m}$  and widths of about 3  $\mu\text{m}$ ) also generated during in vivo experiments from various groups. George and Vashishth [38] generated linear microcracks of up to 300  $\mu\text{m}$  length and 8  $\mu\text{m}$  width by conducting fatigue tests on human cortical bone specimens. Burr et al. [2, 22] and Kennedy et al. [39] investigated microcracks with lengths of 200–800  $\mu\text{m}$  and widths of 6–10  $\mu\text{m}$  in canine femurs and human bones, respectively. Diab and Vashishth [5] created microcracks of about 200  $\mu\text{m}$  length and about 3  $\mu\text{m}$  width, whereas Wasserman et al. [40] described microcracks of up to 1,000  $\mu\text{m}$  length and about 7  $\mu\text{m}$  width in human bone. Additionally, introduction of linear microcracks produced areas of diffuse damage in our samples, which normally also arise in in vivo experiments [2, 41, 42]; but osteoclasts did not show any preference to resorb these areas either (data not shown). Beyond those visible microcracks, it could be possible that tiny cracks, invisible under the bright field microscope, may be present also; however, these could not be investigated.

However, introduction of microdefects, such as microscratches and microcracks, did not influence the resorption behavior of osteoclasts, as revealed by image analysis and statistical evaluation. Furthermore, the physical/architectural presence of microcracks does not have any potential to initiate or to deflect osteoclastic resorption progression in a pure osteoclast culture system, lacking communication signals from other cells. Besides, it has been reported that microcracks, in addition to their talent to initiate remodeling, have the potential to steer already existing basic multicellular units (BMUs). Martin [43] described a directed movement of osteoclasts in BMUs toward microcracks and assumed involvement of the osteocytic system in this process. Our observations are also in line with the ideas of Da Costa Gomez et al. [44], who showed that targeted bone remodeling in racehorses is not exclusively connected to microcracks. Nevertheless, targeted bone remodeling is described in the literature to be *the* in vivo tool for removal of microcracks in the skeleton. Burr et al. [10, 22, 23] showed in dog long bones that microcracks are associated more often with resorption spaces after loading than expected randomly,

thus confirming that this microdamage could initiate bone remodeling. Experimental studies in canine bone after cyclic loading also showed increased remodeling events associated with microcracks [10]. Even in human bone it was demonstrated that cracks are associated with higher cortical remodeling [39]. Furthermore, Herman et al. [11] and Bentolila et al. [21] showed that microcracks in cortical bone of lengths of approximately 200  $\mu\text{m}$  and widths up to 5  $\mu\text{m}$  (dimensions also used in our experiments) are associated with resorption spaces and that microdamage has the potential to activate intracortical remodeling in rats, which normally do not have intracortical bone remodeling. Thus, these data confirm that targeted bone remodeling in vivo happens due to microcracks. Moreover, our data show that osteoclasts per se do not possess a toposensitivity to recognize microcracks due to their physical presence, which further indicates that targeted resorption is based on the communication of osteoclasts with other cells, most likely with osteocytes. Noble et al. [17] suggested that microcracks finally always destroy some osteocytes, which then induce a cascade ending in osteoclastic activity at those sites. Thus, living osteocytes seem to send a kind of nonresorbing signal to osteoclasts, and when this signaling stops osteoclasts can resorb. Beyond osteocytes, also osteoblasts play an important role in the resorption process and osteoclastogenesis, via direct and indirect cell–cell communications. Osteoblasts are connected to osteocytes via gap junctions [45], and it is suggested that osteoblasts sense osteocyte cell death via gap junctional intercellular communication, followed by expression of adhesion molecules affecting migration of osteoclast precursors [34]. At the same time, soluble and membrane-bound factors from osteoblasts affect the differentiation of osteoclast precursor cells as well as the osteoclastogenic phenotype [46].

Beyond these records, we showed that osteoclasts per se do not possess a toposensitivity which would allow them to recognize the physical presence of microcracks. Our data complete existing in vivo data and shed new light on the complex process of targeted bone remodeling by excluding one possible mechanism in defect recognition, namely, osteoclastic toposensitivity. This emphasizes the key role of osteocytes and, perhaps, osteoblastic lining cells in this process. Thus, in case of targeted bone remodeling, osteoclasts need to be guided to resorption places via biochemical information or via direct cell–cell contact from other cell types since they do not recognize microcracks due to their physical presence.

## Conclusion

Osteoclasts are deflected by large discontinuities, but they do not possess a toposensitivity which would allow them to

directly react to the physical presence of architectural defects like microcracks.

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