

## RESEARCH ARTICLE

# Tumor Formation of Adult Stem Cell Transplants in Rodent Arthritic Joints

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### Abstract

**Purpose:** While imaging matrix-associated stem cell transplants aimed for cartilage repair in a rodent arthritis model, we noticed that some transplants formed locally destructive tumors. The purpose of this study was to determine the cause for this tumor formation in order to avoid this complication for future transplants.

**Procedures:** Adipose-derived stem cells (ADSC) isolated from subcutaneous adipose tissue were implanted into 24 osteochondral defects of the distal femur in ten athymic rats and two immunocompetent control rats. All transplants underwent serial magnetic resonance imaging (MRI) up to 6 weeks post-transplantation to monitor joint defect repair. Nine transplants showed an increasing size over time that caused local bone destruction (group 1), while 11 transplants in athymic rats (group 2) and 4 transplants in immunocompetent rats did not. We compared the ADSC implant size and growth rate on MR images, macroscopic features, histopathologic features, surface markers, and karyotypes of these presumed neoplastic transplants with non-neoplastic ADSC transplants.

**Results:** Implants in group 1 showed a significantly increased two-dimensional area at week 2 ( $p=0.0092$ ), 4 ( $p=0.003$ ), and 6 ( $p=0.0205$ ) compared to week 0, as determined by MRI. Histopathological correlations confirmed neoplastic features in group 1 with significantly increased size, cellularity, mitoses, and cytological atypia compared to group 2. Six transplants in group 1 were identified as malignant chondrosarcomas and three transplants as fibromyxoid sarcomas. Transplants in group 2 and immunocompetent controls exhibited normal cartilage features. Both groups showed a normal ADSC phenotype; however, neoplastic ADSC demonstrated a mixed population of diploid and tetraploid cells without genetic imbalance.

Fanny Chapelin and Aman Khurana contributed equally to this work.

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**Conclusions:** ADSC transplants can form tumors *in vivo*. Preventive actions to avoid *in vivo* tumor formations may include karyotyping of culture-expanded ADSC before transplantation. In addition, serial imaging of ADSC transplants *in vivo* may enable early detection of abnormally proliferating cell transplants.

**Key words:** Magnetic resonance imaging, Stem cell therapy, Chondrosarcomas, Fibromyxoid sarcomas, Osteochondral transplants, Malignant tumors *in vivo*

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## Introduction

The field of regenerative medicine is rapidly expanding, with currently over 4000 ongoing clinical stem cell trials for the treatment of over 1400 medical conditions [1]. Adipose-derived stem cells (ADSC) have been recently introduced as a new source for bone and cartilage regeneration in patients with sports injuries or degenerative arthritis [2–4]. Subcutaneous fat depots, the source for these cells, are abundant and easily accessible, thereby providing a potentially unlimited reservoir for retrieval of adult stem cells [5]. By comparison, bone marrow-derived mesenchymal stem cells (MSC) have to be harvested by invasive bone marrow aspiration and may provide a lower yield of stem cells per harvest [6]. Although advantages and disadvantages of both cell types are discussed controversially [7, 8], several studies have shown that ADSC in growth factor-enriched scaffold could provide similar or better chondrogenic and osteogenic differentiation outcomes when compared to MSC [6–9]. Thus, ADSC have become an attractive source for matrix-associated stem cell implants in arthritic joints, with the aim to regenerate bone and cartilage defects [2, 10–12].

While studying matrix-associated stem cell implants in rodents with magnetic resonance imaging (MRI), we noticed that some of our ADSC transplants formed locally destructive tumors. Tumor formations of pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells have been reported previously in both rodent models and humans [13–15]. Other investigators reported bone marrow MSC/ADSC transforming into carcinoma-associated fibroblasts when cross-contaminated with or co-injected with tumor cells/growth factors [16–18]. To the best of our knowledge, tumor formations after *in vivo* transplantation of transformed adult ADSCs have not been reported so far. To evaluate the cause of the observed tumorigenesis, we compared the imaging characteristics, macroscopic and histopathologic features, phenotypes and karyotypes of ADSC transplants that led to tumor formation with non-neoplastic ADSC transplants that resulted in cartilage defect regeneration.

## Materials and Methods

### *Animal Model and ADSC Implantation*

The study was approved by our institutional animal care and use committee. Studies were performed in 12 6–8-week-old male Sprague Dawley rats, including 10 athymic rats, and 2

immunocompetent controls. Athymic rats were chosen to avoid immune rejection of allogeneic transplants and to enable comparisons with prospective human stem cell implants. ADSC were extracted from a donor rat using established procedures [5, 19]. ADSC were then expanded in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), and 100 I.U./ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere. At 80–90 % confluency, the ADSC were trypsinized, the viability was calculated with a trypan blue test, and either cultured further or used for experiments.

Approximately  $7.5 \times 10^5$  ADSC in agarose scaffold were implanted into osteochondral defects of the bilateral distal femurs of 12 6–8-week-old male Sprague Dawley rats. Surgeries were performed under sterile conditions and isoflurane anesthesia by an experienced animal surgeon: a circular osteochondral defect (2 mm diameter, and 1.5 mm depth) was created in the inter-trochlear groove of the femur using a micro-drill (Ideal, Sycamore, IL), and ADSC implants were introduced into the defects. The implant location and consistency was confirmed visually and by gentle palpation with forceps, and the skin incision was closed with Dermalon 6-0 monofilament sutures. Potential post-surgical pain was controlled by subcutaneous administration of buprenorphine (0.05 mg/kg).

### *MRI of ADSC Transplants*

All rats underwent MRI on a 7T animal MR scanner (General Electric-Varian "microSigna 7.0" collaboration). These scans were obtained directly after ADSC transplantation as well as at 2, 4, and 6 weeks post-transplantation. Animals were anesthetized with 1.5–2 % isoflurane and placed supine with knee in an extended position. A custom-built single-channel transmit/receive partial birdcage radio-frequency coil with an inner diameter of 4 cm was placed around the animal's knee for imaging. Sagittal MRI images of both knee joints were obtained with fast spin-echo (FSE) sequences with a repetition time of 3000 ms, echo time of 30 ms, field-of-view of  $2.5 \times 2.5$  cm, a matrix of  $256 \times 256$  pixels, a slice thickness of 0.5 mm, and 16 acquisitions.

The two-dimensional area of the ADSC transplants on the sagittal imaging plane that covered the largest dimension of the transplant was measured as length  $\times$  width on serial MRI images using a DICOM image processing software (Osirix,

Pixmeo, Geneva, Switzerland). The average growth rate was determined by dividing the difference in area of the transplants over 6 weeks by the number of weeks:  $(\text{Area (week 6)} - \text{area (week 0)})/6 = \text{growth rate (cm}^2/\text{week)}$ .

### Histopathology

Animals were sacrificed after the last MRI procedure, knee joints were explanted, and macroscopic specimen were evaluated for signs of invasive tumor growth, as evidenced by extension of the transplant beyond the normal joint surface and macroscopic invasion of adjacent bone or joint components. Joint specimens were then dissected and placed in Cal-ex II (Fisher Scientific) for 5–8 days to fix and decalcify the tissue simultaneously. The specimens were dissected para-sagittally, dehydrated through graded alcohol washes, and embedded in paraffin. Five-micrometer thick tissue slices were stained with hematoxylin and eosin (H&E) for microscopic evaluation of the ADSC transplants. Two experienced pathologists (KH, DG) determined the histopathologic size, nuclear:cytoplasm ratio, the number of mitoses, and presence or absence of microscopic invasion of adjacent bone or soft tissues. Based on the criteria above and additional clinical standard criteria for the classification of bone and soft tissue tumors [20], the pathologists classified the implants as neoplastic or non-neoplastic and within the neoplastic group, further determined the histopathological tumor type.

### ADSC Phenotyping

Like bone marrow MSC, ADSC express CD29, CD44, CD71, CD90, CD105/SH2, SH3, and the widely recognized stem cell marker STRO-1. ADSC do not express CD31, CD45, and CD106 [21]. ADSC underwent comprehensive phenotyping before their transplantation by our collaborators from the custom technology team at BD Biosciences (San Diego, CA). Phenotyping analysis included CD44H-FITC, CD31-PE, CD29-A647, and CD45-V450 antibodies, evaluated with the BD FACS Canto II flow cytometer. Details of these reagents are listed in Table 1. Compensation was set up using BD Comp Beads. Data were quantified using BD FACS Diva software (BD Biosciences, San Jose, CA, USA) with gate settings for the major population of FSC (cell size)/SSC (granularity) plot. The gated cell types were visualized on 2-parameter dot plots and the percentages of specific populations were calculated.

**Table 1.** Antibodies used for ADSC phenotyping analysis

Specificity	Clone	Isotype	Format
1	CD44H	OX-49	Ms IgG2a, $\kappa$ FITC
2	CD31	TLD-3A12	Ms IgG1, $\kappa$ PE
3	CD29	Ha2/5	Hamster IgM, $\kappa$ A647
4	CD45	OX-1	Ms IgG1, $\kappa$ V450

### Chromosome Analysis

ADSC used for the implants were retrospectively evaluated by the Cytogenetics facility at Stanford. The ADSC cell line was harvested by standard cytogenetic methodology of mitotic arrest, hypotonic shock, and fixation with 3:1 methanol-acetic acid. Chromosome slide preparations were stained by G-banding and classified by the standard Norway rat karyotype [22].

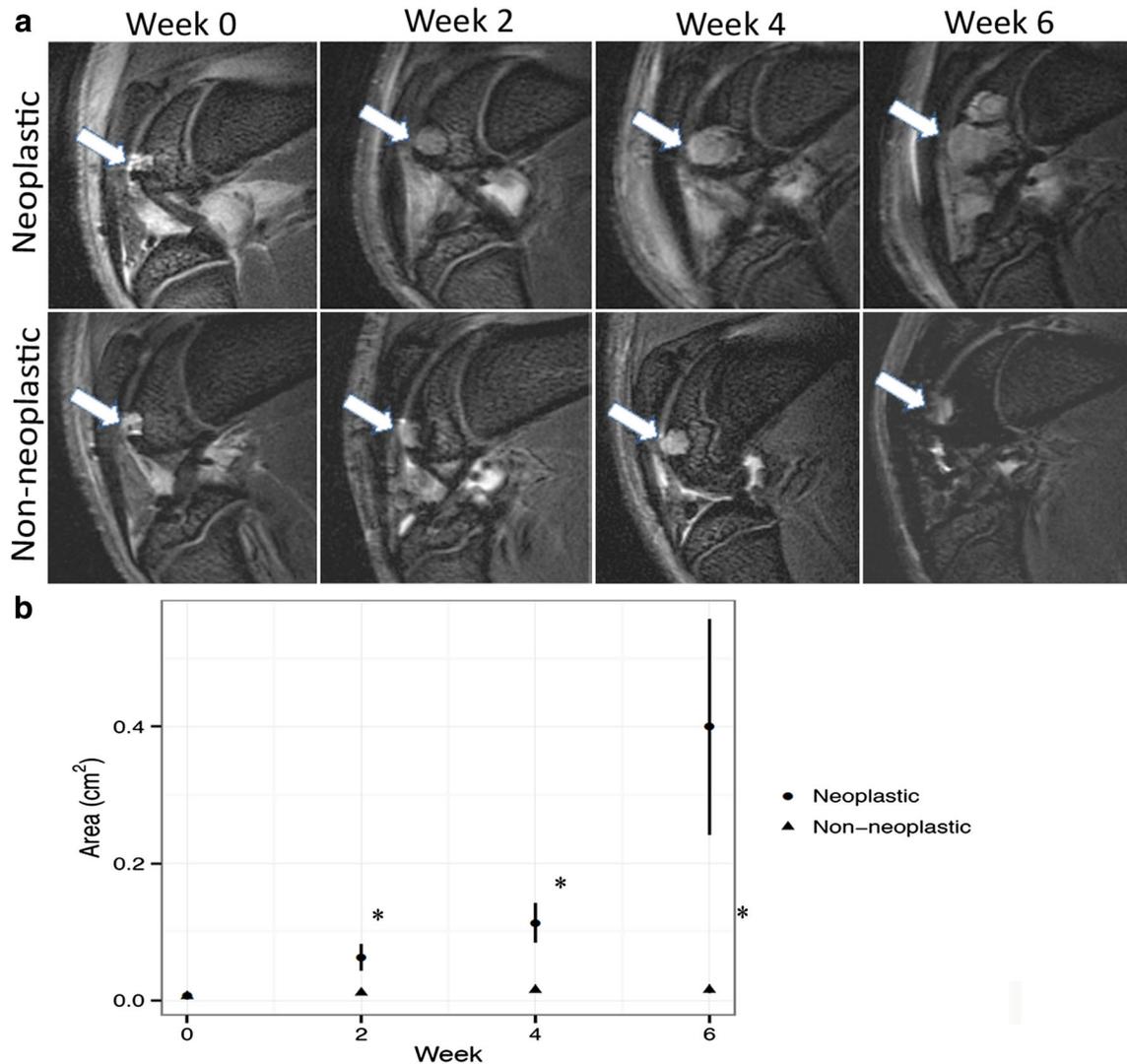
### Data Analyses

Based on visual assessment of MRI images by two independent observers (AK, FC), ADSC transplants were divided into two groups: group 1 (presumed neoplastic) implants, that markedly increased in size over time, caused local bone destruction and extension into the joint and group 2 (presumed non-neoplastic) implants that did not or only minimally (not more than 1 mm) increased in size over time and did not show any imaging signs of local bone destruction. The two-dimensional area of all transplants in the sagittal plane was measured on serial MRI images at week 0, 2, 4, and 6. Results were compared between group 1 and 2, using a *t* test with unequal variances. Similarly, the number of cells, mitoses and cell atypias per high power field (hpf) were compared for significant difference between both groups using a *t* test. A *p* value of less than 0.05 was considered significant.

## Results

### MRI Features of Neoplastic and Non-Neoplastic ADSC

In athymic rats, 9 cell transplants increased in size in the implant area (group 1) and 11 did not (group 2). In immune-competent rats, no cell transplant increased in size *in vivo*. Implants in group 1 showed a significantly increased area in MRI images at week 2 ( $p=0.0092$ ), 4 ( $p=0.003$ ), and 6 ( $p=0.0205$ ) compared to week 0. In addition, implants in group 1 demonstrated signs of local bone destruction at week 4 and 6 as well as signs of joint invasion beyond the transplant site at week 6. Conversely, implants in group 2 showed no significant changes in size of the implant at week 2, 4, and 6 ( $p>0.05$ ) and no signs of bone or joint destruction at any time (Fig. 1). The mean growth rate of neoplastic ADSC transplants (group 1) was  $0.061 \pm 0.0716 \text{ cm}^2/\text{week}$  (range 0.0059–0.2137) as compared to  $0.0019 \pm 0.002 \text{ cm}^2/\text{week}$  (range 0.0004–0.0027) for non-neoplastic transplants (group 2). These data were significantly different ( $p=0.0147$ ). Four control transplants in two immunocompetent recipients showed no increase in the implant area over 4 weeks and no signs of bone or joint destruction at any time (see Supplemental Fig. 1a–b in Electronic Supplementary Material (ESM)).



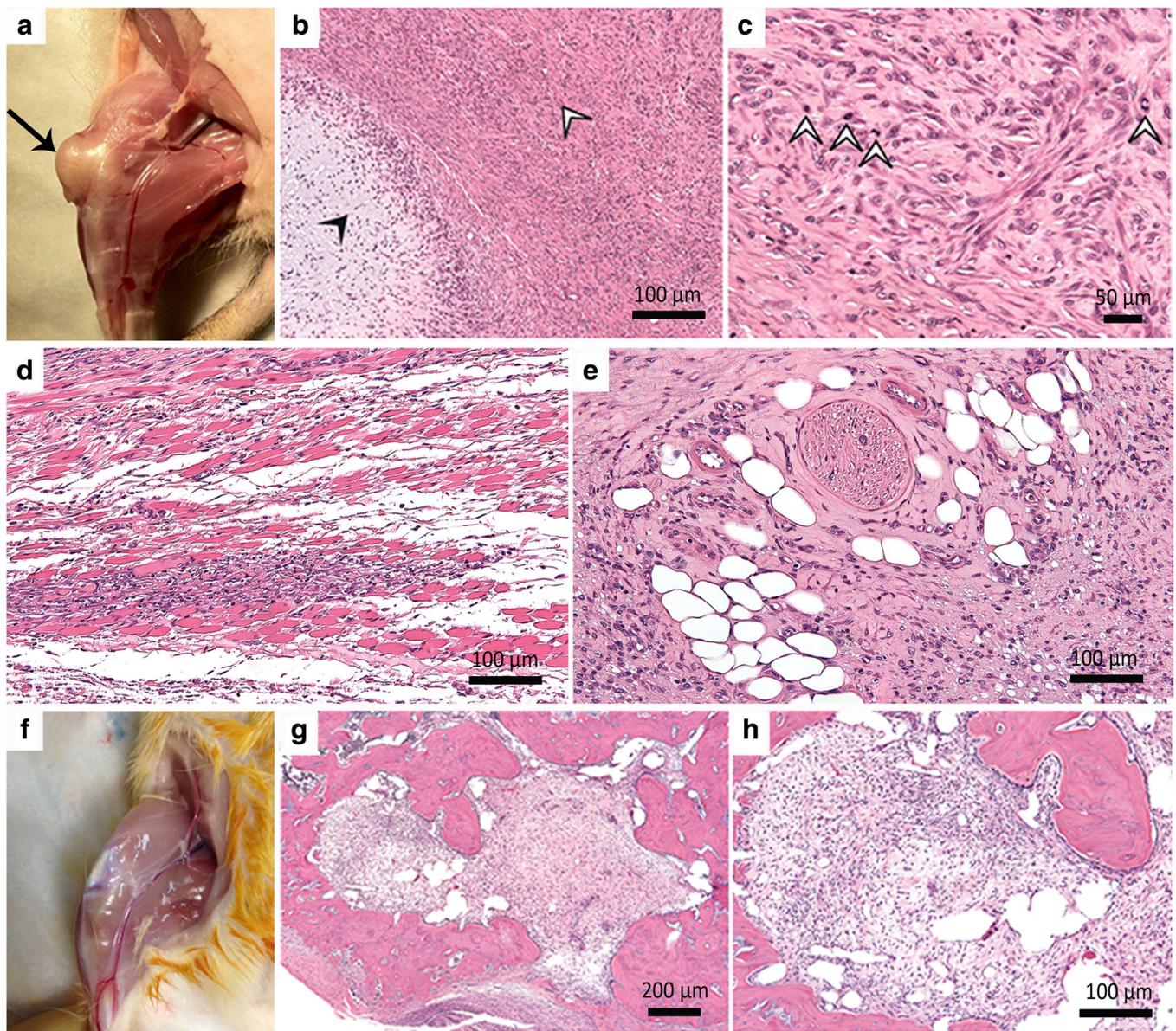
**Fig. 1** Magnetic resonance findings for neoplastic and non-neoplastic transplants. **a** Sagittal T2-weighted FSE images (TR/TE = 3000 ms/30 ms) of ADSC transplants in cartilage defects of distal femurs (arrows): neoplastic ADSC implants show significant increase in size over 6 weeks, whereas non-neoplastic ADSC implants did not expand. **b** Corresponding size of neoplastic and non-neoplastic ADSC transplants at different time points after implantation, displayed as mean transplant area with standard errors. A single asterisk indicates significant differences between neoplastic and non-neoplastic ADSC transplants ( $p < 0.05$ ).

### *Histopathologic Features of Neoplastic and Non-Neoplastic ADSC*

Gross examination of the knee joints of animals in group 1 revealed palpable masses in the distal femur, which were large enough to impair the mobility of the knee joint. Postmortem macroscopic evaluation revealed highly vascular, expansive and locally destructive masses of the distal femur with multiple sites of necrosis, including the transplant site and extending into adjacent bone and joint components (Fig. 2a). Of the nine neoplastic transplants, four occurred in the bilateral knees of the same rats and five others occurred unilaterally in one knee, while the other knee demonstrated normal transplants. The animals were therefore not responsible for providing a conducive

environment for tumor growth; otherwise, tumor formation would always have occurred in both knees of affected animals.

Histopathologic examination of the transplants in group 1 revealed malignant chondrosarcomas in six cases and fibromyxoid sarcomas in three cases. The chondrosarcomas were characterized by mixtures of myxoid and hyaline cartilage, foci of extra-osseous soft tissue invasion, and encroachment upon peripheral nerve fibers (Fig. 2b, c, d, e). Fibromyxoid sarcomas presented with overgrowth of loose connective tissue within the area of the previous implant with typical herringbone patterns and cell pleomorphism, which extended into the marrow space but did not infiltrate the cortical bone. The sarcomas demonstrated a wide range of histological signs of tumor proliferation and atypia. Chondrosarcomas exhibited high cell



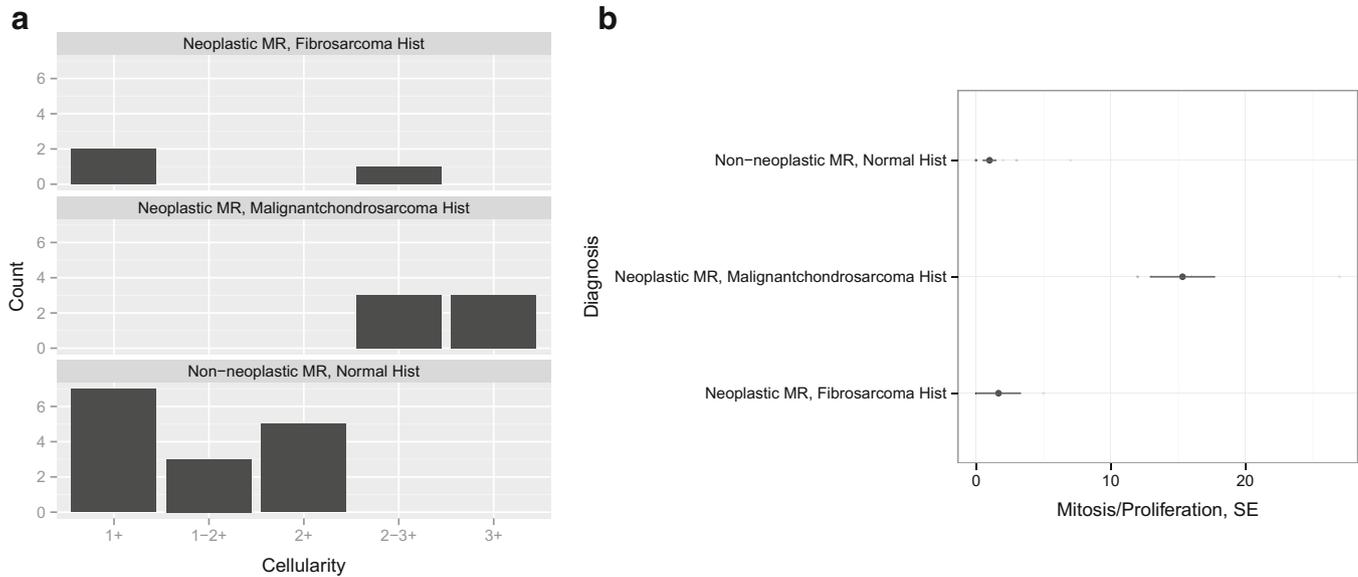
**Fig. 2** Macroscopic and microscopic features of neoplastic and non-neoplastic ADSC transplants. **a** Neoplastic ADSC transplants present as an expansile and locally destructive mass (arrow). **b** Histopathological features of the malignant chondrosarcomas at  $\times 100$  and **c**  $\times 400$  include myxoid production (closed arrowhead) and hyaline cartilage (open arrowheads). **d** The malignant cells infiltrate adjacent skeletal muscle ( $\times 200$ ), and **e** adipose tissue with encroachment upon a nerve ( $\times 200$ ). **f** Non-neoplastic ADSC transplants do not extend beyond the transplant site. H&E stains of non-neoplastic transplants at **g**  $\times 40$  and **h**  $\times 400$  exhibit a well-defined cell implant with chondrogenic cells surrounded by unremarkable native bone.

density, cytologic atypia, and an average of  $15.33 \pm 5.4$  mitoses per hpf, while fibrosarcomas exhibited intermediate cell density, cytological atypia, and an average of  $1.67 \pm 2.9$  mitoses per hpf (Fig. 3).

Stem cell transplants in the non-neoplastic group (group 2) did not show macroscopic signs of tumor formation and no evidence for abnormal tissue proliferation outside the transplant site (Fig. 2f). Contrary to group 1, all transplants in group 2 exhibited low cell density (paucicellular), no or minimal cell atypia, and  $1.11 \pm 2.02$  mitoses per hpf. (Figs. 2g, h, and 3). These criteria were significantly

different between groups 1 and 2 ( $p=0.0008$ ). Neoplastic transplants formed highly proliferative tumors while cells in non-neoplastic transplants apparently stopped proliferating and differentiated into cartilage to repair the osteochondral defect (Fig. 2g, h) [23].

Gross examination and macroscopic evaluation of the knee joints of immunocompetent control animals showed no evidence for mass effect (see Supplemental Fig. 1b in ESM). Immunocompetent transplants demonstrated low cell density, no cell atypia, and 0 mitoses per hpf (see Supplemental Fig. 1c and d in ESM).



**Fig. 3** Histopathological classification of neoplastic and non-neoplastic transplants and MRI correlation. Normal and malignant ADSC implants were quantitatively compared based on **a** cellularity (1+ = paucicellular, 2+ = moderately cellular, 3+ = highly cellular) and **b** the proliferation rate per high power field, hpf ( $\times 400$  magnification). Malignant transplants were defined based on high cellularity and high mitosis rate. Non-neoplastic transplants exhibited low mitosis rates that were the result of cartilaginous proliferation.

### ADSC Phenotyping

FACS analysis of ADSC before implantation into animals and FACS analyses of neoplastic and non-neoplastic ADSC showed that all samples were positive for CD29 and CD44H markers and negative for CD31, which is consistent with the phenotype of ADSC [19, 21, 24] (Tables 1 and 2, Fig. 4). These findings confirm that evaluation of phenotypic markers alone is not sufficient to assess the potential of cellular tumorigenicity before or after stem cell implantation. Of note, non-neoplastic ADSC contained a small population of CD45+ cells, which was not noted in neoplastic cell samples. These cells most likely represented contaminating leukocytes.

### Chromosome Analysis

Chromosome analysis of tumor-forming ADSC demonstrated a mixed population of male diploid (42, XY) and tetraploid (84, XXYY) cells without obvious structural rearrangement or genetic imbalance (Fig. 5). By contrast, non-neoplastic ADSC demonstrated a normal male diploid

karyotype only. These findings suggest that normal ADSC spontaneously transformed into neoplastic ADSC, which are easily distinguishable by aberrant chromosomal numbers.

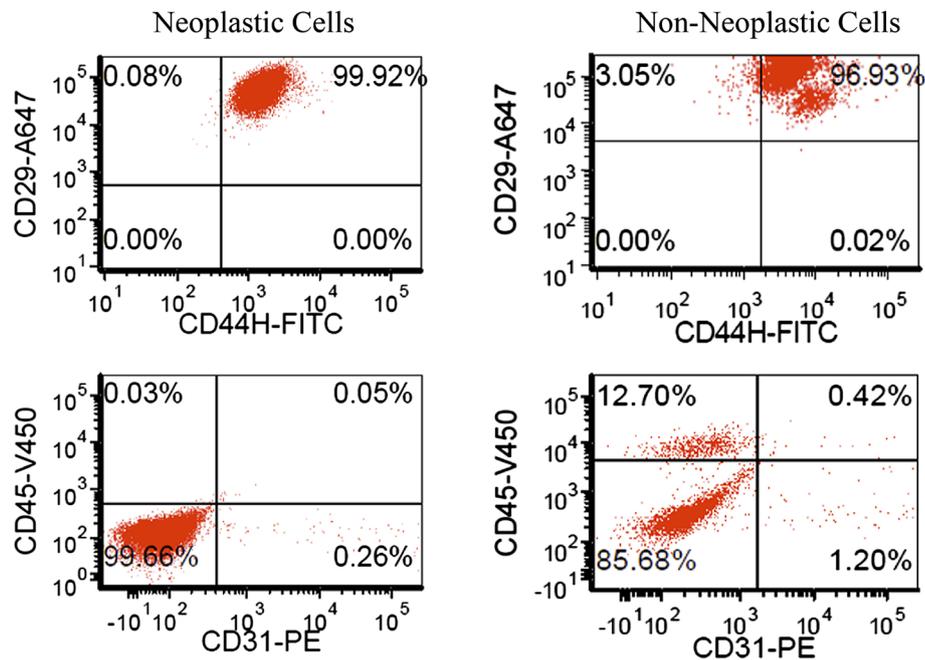
### Discussion

Our data showed neoplastic transformation of ADSC transplants in a preclinical model of arthritic joints of recipient rats. Nine out of 24 ADSC transplants developed into invasive tumors. Based on our karyotyping data, the most likely cause for neoplastic transformation of our ADSC is a genetic transformation during cell expansion in cell culture. This is in accordance with other reports of genetic or phenotypic transformation of ADSC [25] and bone marrow-derived MSC [26–30] during *ex vivo* expansion, possibly due to augmented chromosome instability associated with dysregulation of telomere activity and cell cycle-related genes [27–29, 31]. There was a trend that later passage MSC induced tumors more frequently compared to earlier passage MSC [27, 28]. In our studies, since the chromosome analyses were performed of cells before implantation and not cells extracted from the tumors, we have evidence that some of the cells in the *ex vivo* culture transformed into the abnormal tetraploid karyotype. We presume that some of the implants contained these abnormal cells and others did not (or to a much lesser extent). The transformed cells outgrew normal ADSCs in some of the transplants and then were able to develop into fibromyxoid sarcomas and chondrosarcomas.

Yet, other investigators reported that relatively high serum concentrations (10–15 %) in cell culture medium could markedly increase the proliferation rate of MSC, possibly mediated by cytokines and growth factors in serum, such as

**Table 2.** Surface markers of normal and neoplastic ADSC

% Expression	Non-neoplastic ADSC	Neoplastic ADSC
CD29+ CD44H+	96.93	99.92
CD45+ CD29+	13.12	0.08
CD45– CD29+	86.88	99.92
CD31+ CD29+	1.6	0.31

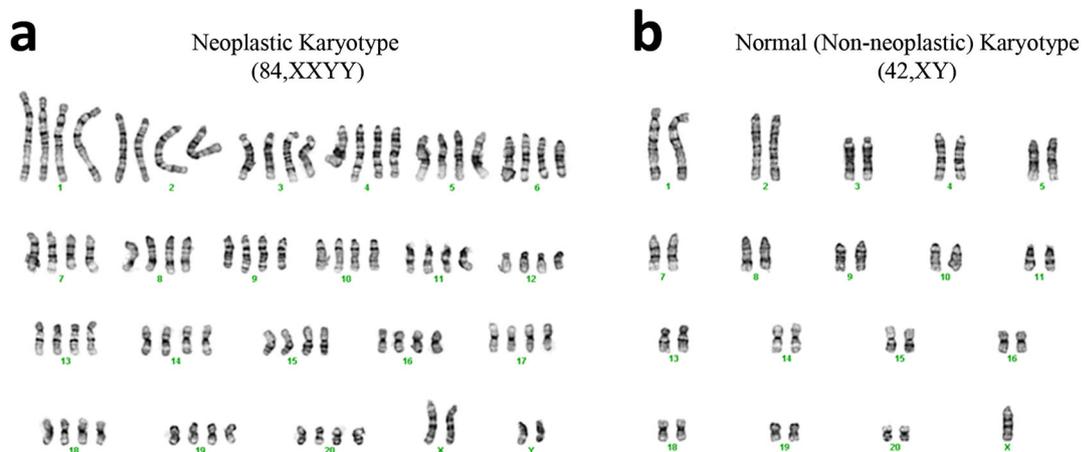


**Fig. 4** Neoplastic and non-neoplastic cells' CD marker expression. Dual axis dot plots show similar expression for neoplastic and non-neoplastic transplants: CD29 and CD44H positive and CD31 negative. Phenotypic markers are not sufficient to discriminate the presence of neoplastic cells in a future transplant.

platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [32, 33]. Since we also used 10 % serum containing media, this mechanism might have contributed to our results. Conversely, other authors did not observe phenotypic or genotypic alterations of stem cells in long-term culture. Bernardo et al. reported that BM-MSC could be cultured for 44 weeks without losing their phenotypical and functional characteristics [34]. However, it is noteworthy that these MSC did apparently not reach senescence, even after 25 passages.

Suggested preventive actions include the use of serum-free media with added proteins [35, 36], ADSC phenotyping

before their transplantation or direct use of harvested ADSC, without any cell expansion [28, 31]. Serum-free media provide less efficient ADSC expansion, thereby counteracting the desired outcome of increasing ADSC quantities. In addition, the effects of various added proteins and growth factors and interactions of scaffolds and growth factors/proteins in scaffold with embedded stem cells are not fully elucidated regarding their impact on ADSC phenotype and karyotype [23, 32, 37–40]. In our case, an effect of the scaffold on neoplastic transformation seems unlikely, since ADSC embedded in the same agarose scaffold either engrafted



**Fig. 5** Chromosome analysis of **a** neoplastic and **b** non-neoplastic ADSC. Neoplastic cells show a mixed population of normal male diploid (42, XY) and aberrant tetraploid (84, XXYY) cells without obvious structural rearrangement or genetic imbalance while non-neoplastic ADSC show a normal karyotype.

normally or started to form tumors. Avoiding *ex vivo* stem cell expansion appears to be the safest approach, and is implemented in several clinical applications of ADSC mediated bone and cartilage repair [41–44]. ADSC expansion *in vitro* prior to delivery to the patient is nonetheless common in other conditions such as Buerger’s disease [45, 46], where cells are cultured for several weeks. Culture media may include FBS followed by human serum or enriched serum-free media for clinical use or 5 % human serum alone. ADSC cells may be from a single donor [45] for each recipient or multiple donors [46], which may complicate careful analysis of cell products and safety.

Current literature is lacking for *in vivo* neoplastic differentiation of proven non-neoplastic MSC/ADSC, but Wislet-Gendebien et al. retrospectively noted that one of the clones of the neural crest stem cells derived from bone marrow aspiration resulted in tumorigenesis *in vivo* after long-term *in vitro* passaging [47]. This specific clone surprisingly had the highest ability to differentiate into neuronal cells (*in vitro*), and also showed a very high rate of proliferation after injection into mice striatum, when compared to the other clones. Upon further transcriptomic analyses, this clone highlighted numerous cell cycle checkpoint modifications and chromosome 11q downregulation, which suggested *ex vivo* neoplastic transformation. This was probably missed due to satisfactory neuronal differentiation, thus highlighting the need for phenotypic, functional, and genetic assays prior to stem cell transplantation. Ning et al. also described an aberrant *ex vivo* expanded ADSC cell line, very similar to our cell type, which demonstrated an increased proliferation rate, a hypertriploid karyotype and abnormal endothelial cell surface markers (similar to angiosarcomas).

Our data suggests that the compromised immune system of our athymic recipients supported tumor formation, because tumors in immunocompromised hosts showed a higher number of mitoses, *etc.* than transplants observed in immunocompetent recipients. We hypothesize that an intact immune system may be able to eliminate few transformed cells *in vivo*. We used athymic rats in order to exclude rejection and enable future extension of our studies to human ADSC. Tumor formation of stem cell transplants in immunodeficient and immunocompetent recipients has been reported previously [27–29, 48, 49]. Miura et al. demonstrated that *ex vivo*-transformed bone marrow MSC caused malignant fibrosarcomas *in vivo* [27]. Other authors described no secondary or distant tumors in any organs in nude/athymic mice after intravenous/subcutaneous injection of transformed MSC [25, 26]. Interestingly, Li et al. reported development of fibrosarcomas after subcutaneous implantation of *ex vivo*-transformed MSC in immunocompetent hosts. This resulted in recruitment of host bone marrow-derived cells and fusion of these cells with transformed MSC, ultimately restoring the non-malignant phenotype. These “fused” cells were unable to form secondary tumors in mice and could not be propagated extensively in culture [48].

It has been argued that *in vivo* tumor formations may be only observed in animal models but not in clinical studies

[50, 51]. Rodent cells are known to be prone to spontaneous transformation when expanded in culture under standard conditions [52–54], while dedifferentiation of human stem cells in cell culture has not been described so far. Observations of tumor formations due to stem cell transplants in patients are starting to emerge in clinical trials. Amariglio et al. reported the first case of a multifocal brain tumor in a 13-year-old patient 4 years after neural stem cell transplantation [55]. Secondary tumors can develop after allogenic or autologous hematopoietic stem cell transplantation (SCT) with reported cumulative incidence of solid cancers following allogeneic SCT ranges from 1.2 to 1.6 % at 5 years, from 2.2 to 6.1 % at 10 years, and from 3.8 to 14.9 % at 15 years post-transplantation [56]. SCT patients usually undergo an immunosuppressive regimen prior to and post-transplant to reduce graft *versus* host disease incidence [56–58]. Majority of the secondary tumors are non-squamous cell carcinomas with radiation as the single most important risk factor [57]. The non-radiation-related secondary tumors are usually squamous cell carcinomas of the skin and mucosa, which are strongly linked to chronic graft *versus* host disease (GVHD) [58]. Thus, most secondary malignancies demonstrate a multifactorial etiology but a clear and direct relationship to implanted stem cells is yet to be elucidated. There are currently more than 115 ongoing clinical stem cell trials involving ADSC [1, 59–62], but tumor formation of adult ADSC in patients has not been described so far. However, secondary tumor formations may only occur years or decades after the transplantation [55]. Thus, diagnostic tools to minimize this important complication are critically needed.

We recognize several limitations to our study. The correlation of chromosomal analysis with tumor formation was performed retrospectively, limiting our ability to establish causality. Comprehensive analysis of each cell culture batch prior to implantation may have helped to further decipher mechanisms of tumorigenesis. In addition, the two immunocompetent animals have little statistical value but provide incidental evidence that an intact immune system might have protective effects. Nonetheless, we believe that this data may foster further discussion on *ex vivo* and *in vivo* factors that can lead to tumor growth of adult cell transplants in immunocompromised *versus* immunocompetent recipients and drive future research towards better understanding of carcinogenesis of therapeutic cell transplants.

In conclusion, our data showed that adult ADSC in injured joints can form malignant tumors *in vivo* and MRI is a non-invasive test that can detect tumor formation of stem cell transplants as early as 2 weeks in animal models based on abnormal growth of the transplant. Thus, MRI might be useful to detect abnormal *in vivo* proliferation of transplanted stem cells. While ADSC show great promise for various tissue regeneration applications, caution must be taken regarding the tumorigenic potential of these cells. A more comprehensive understanding of ADSC biology and

ADSC-host interactions is critically needed. A standardized protocol for genotyping of *ex vivo* expanded ADSC prior to their implantation as well as non-invasive imaging techniques for *in vivo* monitoring of the transplanted cells will greatly enhance the safety of ADSC-mediated tissue regeneration procedures.

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#### Compliance with Ethical Standards

#### Conflict of Interest

The authors declare that they have no conflict of interest.

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