The CD24\textsuperscript{hi} smooth muscle subpopulation is the predominant fraction in uterine fibroids

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Submitted on December 31, 2013; resubmitted on February 20, 2014; accepted on February 26, 2014

\textbf{ABSTRACT:} Uterine fibroids are the most common gynecological tumors affecting women in their reproductive age. Despite this high incidence the pathogenesis of fibroids is widely unsolved. Whereas formerly only imbalances in hormonal levels were considered to account for tumor development, the identification of genetic changes likely to affect myometrial stem cell reservoirs provided a novel approach to fibroid genesis. Here, we identified a certain subset of cells by the surface marker CD24 with increased abundance in fibroids compared with myometrial tissue. Fibroid cells expressing CD24 shared certain features of immature or progenitor-like cells such as quiescence, reduced expression of smooth muscle differentiation markers and elevated expression of genes involved in the wingless-type (WNT)-pathway such as beta-catenin.

In addition, a positive correlation between CD24 and wingless-type family member 4 (WNT4) expression was observed in uterine fibroids with mediator subcomplex 12 gene (MED12) mutations. Our findings suggest that cells highly expressing CD24 represent a type of immature smooth muscle progenitor cells. Their accumulation might be driven by disturbed differentiation processes caused by genetic changes possibly involving MED12 mutations or high mobility group AT-hook (HMGA)2 rearrangements.

\textbf{Key words:} leiomyoma / cancer

\textbf{Introduction}

Uterine fibroids are a common health issue of women in their reproductive age, with incidences reported from 30—70\% (Day Baird et al., 2003; Zimmermann et al., 2012). Although fibroids are of benign nature, they often cause symptoms such as infertility, recurrent miscarriage and extensive bleeding. First demonstrated based on their unique isoenzyme pattern (Townsend et al., 1970) and X-inactivation studies (Canevari et al., 2005; Cai et al., 2007; Mashal et al., 1994), a clonal origin of fibroids was reported recently by a more sensitive and reliable method conducted by Holdsworth-Carson et al. (2013). Hence, it could be considered that fibroids arise from a single mutated cell likely having stem/progenitor cell characteristics. To date the exact subpopulation of cells giving rise to fibroids is still unknown.

Somatic stem cells are believed to reside in tissues mostly in a dormant or quiescent state and divide upon activation to maintain tissues homeostasis (Reya et al., 2001; Gargett et al., 2007). This property has been utilized to identify potential stem cells in myometrium or endometrium through their ability to retain proliferation labels such as 5-bromo-2'-deoxyuridine or \textsuperscript{3}H-thymidin thus marking non-proliferative cells (Cervello et al., 2007; Szotek et al., 2007). At present, many types of these stem cells can be prospectively identified in various tissues by a panel of surface markers correlating with stem cell phenotypes (Reya et al., 2001; Dominici et al., 2006; Gargett et al., 2007). However, exact immunophenotypes especially for uterine stem cells are not yet established (Gargett et al., 2007). Where an unequivocal immunophenotype is missing, stem/progenitor cells can alternatively be identified by their ability to extrude the DNA-binding dye Hoechst 33342. These so-called side population (SP) cells—named after their appearance in flow cytometric dot blots—overexpress the ATP-binding cassette transporter G2 which is responsible for the removal of uptaken Hoechst 33342. First described for bone marrow (Goodell et al., 2005), this technique was also adopted to isolate stem/progenitor cells from various other normal tissues (Zuk et al., 2002; Goodell et al., 2005; Goodell, 2005) such as endometrium (Cervelló et al., 2010; Masuda et al., 2010) or different malignancies (Wang et al., 2007; Wu et al., 2007). Recently, subpopulations with SP phenotype were isolated from myometrium and fibroid tissue (Ono et al., 2007, 2012; Mas et al., 2012). In this latter case, they may represent a transformed type of uterine stem or precursor cells likely to represent a tumor-initiating subpopulation (Mas et al., 2012). The exact mechanism...
of this initiation remains mainly unsolved but a recent study points to an indirect paracrine activation of SP cells by smooth muscle cells (Ono et al., 2013). While the SP detection is a favorable method for isolation of cells with stem cell characteristics, an independent surface marker for fibroid or myometrial progenitors has not been published to date.

CD24 is a highly glycosylated anchor protein commonly present on immature cells such as pre-B-cells, muscle stem/satellite cells (Figarella-Branger et al., 1993; Higuchi et al., 1999), neuronal (Poncet et al., 1996) and hepatic precursors (Qiu et al., 2011) or intestinal (von Furstenberg et al., 2011) and adipose (Rodeheffer et al., 2008) stem cells. Since knockout mice are viable without any obvious defects apart from a reduced number of pre-B-lymphocytes CD24 is considered to be mostly a redundant co-stimulatory molecule during B-cell development (Schabath et al., 2006; Fang et al., 2010) and dampener of inflammation (Chen et al., 2009). In pathophysiological conditions, CD24 was identified on various cancer entities, e.g. breast, prostate, ovary, lung (small cell and non-small cell) and colon cancer (Kristiansen et al., 2002, 2003a, b; 2004a, b, 2005). The expression of CD24 in most of these neoplasms is correlated with poor prognosis and its expression is believed to augment metastasis by interactions with P-selectin (Aigner et al., 1998; Friedrichs et al., 2000; Lim, 2005). The study presented here is addressing the expression of CD24 in fibroids. In a previous set of experiments (data unpublished), we observed a significantly higher expression in fibroids compared with myometrial tissue confirming earlier results by various other groups (for review see by Arslan et al., 2005). The preferential expression of CD24 on immature cells in other organs (Fang et al., 2010) and the increasing body of evidence that uterine stem cells, e.g. observed in form of SP cells, might play a pivotal role in fibroid growth (Ono et al., 2007, 2012, 2013) encouraged us to investigate if differences in CD24 expression reflect differences in cell composition. Increased CD24 expression in fibroids may reflect an accumulation of less differentiated CD24+ cells underpinning a possible role of a stem cell driven pathogenesis of uterine fibroids.

**Material and Methods**

**Ethics statement and tissue sampling**

Fibroid specimens were obtained from patients undergoing myomectomy or hysterectomy. All donors were premenopausal and patients with hormonal or immunomodulatory therapies as well as known gynecological comorbidities or malignancies were excluded. Informed written consent was obtained prior to surgery in accordance with the ethics committee vote of the hospitals (Klinikum MIC, Berlin, Germany, Ethics authorization number ETH-08/10-01; Klinikum Auguste-Viktoria, Berlin, Germany, Ethics authorization number Eth-07/1001; Charité Campus Benjamin-Franklin, Berlin, Germany, Ethics authorization number EA1/135/2012). Tissue was transferred to chilled Viaspan organ conservation buffer (Bristol-Myers Squibb, Dublin, Ireland) and used for experiments within 24 h. Fresh-frozen tissue for mutational analysis was from the archives of two of the authors (D.N.M. and J.B.). Sampling was approved by the local ethics committee, and before surgery informed written consent was obtained from all patients. In addition, all samples were taken initially for diagnostic purposes and de-identified before their use in study following the rules of Helsinki declaration.

**Primary cell isolation and cell culture**

Primary cells were isolated as described previously (Markowski et al., 2010; Drosch et al., 2013). After disaggregation, cells were washed with phosphate-buffered saline (PBS) and cultured in medium 199 (Pan Biotech, Aidenbach, Germany) supplemented with 20% fetal bovine serum and ‘antibiotic–antimycotic’ (Invitrogen, Karlsruhe, Germany), alternatively cells were used for fluorescence-activated cell sorting (FACS) or in vivo transplantation.

**Generation of xenografts**

Mice were kept in transparent Macrolon™ cages in a light/dark cycle of 12 h/12 h under pathogen-free conditions in the animal facility of Bayer Pharma AG, Berlin, Germany. All experiments were performed in strict compliance with company, regional and federal guidelines for the use of laboratory animals. The study was approved by the Office for Health and Social Affairs Berlin and the company review board, and all efforts were made to minimize animals’ stress. Xenografts were established from dissociated fibroids (n = 4) using three to five animal per tumor (dependent on cell yield). Grafting procedures was performed as described previously (Drosch et al., 2013). In brief, 3 × 10⁶ disaggregated tumor cells were resuspended in 100 μl matrigel supplemented with basic fibroblast growth factor, epidermal growth factor and insulin and injected subcutaneously in the ‘posterior lateral’ aspect of the trunk (lumbar region) of hormone-supplemented ovariectomized 6–9-week-old scid/bg mice (Taconic, Cologne, Germany). Animals were monitored twice a week and tumor volume was assessed but remained low as reported before (Drosch et al., 2013). For tumor excision animals were sacrificed by cervical dislocation. Excised tumors were either prepared for cryosectioning by immersing in polyvinyl cryoembedding medium (OCT) (Leica Microsystems, Wetzlar, Germany) or fixed with neutral buffered formalin and subjected to paraffin embedding.

**Cell sorting and flow cytometric analysis**

Cells were sorted using a FACSAnA I special sorting system (BD Bioscience, Heidelberg, Germany) equipped with three lasers (405, 488 and 630 nm) and analyzed using the FlowJo Software (Tree Star, Ashland, USA). For sorting dissociated cells from fibroid (n = 9) or myometrial (n = 4) tissue were washed with Hank’s balanced salt solution (HBBS) and resuspended in chilled staining buffer [Ca²⁺ and Mg²⁺ free HBBS containing 1% bovine serum albumin, 10 mM 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethan-sulfonic acid (HEPES) and antibiotic–antimycotic mixture] and stained with antibodies listed in Table I for 20 min at 4°C. Afterwards cells were washed with an excess of staining buffer, resuspended at a concentration of 10⁷ cells/ml and subjected to the sorting system. Compensation was performed with single stained and isotype controls. Dead cells were discrimination by staining with 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen). Cells were sorted into chilled staining buffer and used either for RNA isolation or cell culture. The purity of the sorted cell populations was verified directly after sorting as >95%. For flow cytometric analysis of cultured cells, monolayers were washed with PBS, detached by treatment with TrypLE™ (Invitrogen), washed and stained as described above. After incubation cells were washed in staining buffer and analyzed using FACSAnA I or LSR II systems.

Intracellular staining of Ki-67 in fibroids (n = 5) was conducted after staining of surface markers. Cells were fixed in freshly prepared high-grade 2% neutral buffered formalin (Electron Microscopy Science, Munich, Germany) for 20 min at room temperature (RT). Cells were washed and stained with anti-Ki-67 in permeabilization buffer (HBBS, 1% BSA; 10 mM HEPES, 0.5% Saponine) for 40 min at RT. After repeated washings, cells were analyzed and 100 000 events were recorded.

**Cytogenetic analysis and DNA sequencing**

For correlation analysis a total of 26 fibroids previously analyzed for cytogenetic aberrations or mutations in the MED12 locus were used (see Supplementary data, Table SI). Cytogenetic characterization was performed...
amplify the desired PCR fragment of the genomic template DNA were amplifications 1.

Quencing of amplified DNA as described (Markowski et al., 2012). As a rule, G-bands (GTG) were analyzed based on a resolution of at least 300 bands/haploid set. If necessary, further characterization was performed by fluorescent in situ hybridization (FISH) using three bacterial artificial chromosome clones located distal (3') and proximal (5'), respectively, of the high mobility group AT-hook (HMGA)2 locus as break-apart probe. Probe labeling was performed by nick translation either with SpectrumOrange-dUTP or SpectrumGreen-dUTP (Abbott Molecular, Wiesbaden, Germany). Fifteen microliters of the break-apart probe (concentration: 100 ng/ml) was used per slide. Post-hybridization was performed at 42°C for 2 min in 0.4 x SSC/0.3% NP-40. Interphase nuclei were counterstained with DAPI (0.75 µg/ml). Slides were examined with an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Gottingen, Germany); images of 100 non-overlapping nuclei were captured with a high performance CCD-camera (Visitron Systems, Puchheim, Germany); images of 100 non-overlapping nuclei were captured with an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Goettingen, Germany). Fifteen microliters of the break-apart probe (concentration: 100 ng/ml) was used per slide. Co-denaturation was performed at 85°C followed by over-night hybridization in a humidified chamber at 37°C. Post-hybridization was performed at 42°C for 2 min in 0.4 x SSC/0.3% NP-40. Interphase nuclei were counterstained with DAPI (0.75 µg/ml). Slides were examined with an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Gottingen, Germany); images of 100 non-overlapping nuclei were captured with a high performance CCD-camera (Visitron Systems, Puchheim, Germany) and edited with FISH View (Applied Spectral Imaging, Migdal HaEmek, Israel).

MED12 mutations from fresh-frozen tumors were identified by DNA sequencing of amplified DNA as described (Markowski et al., 2012). For PCR amplifications 1 µg of genomic template DNA was used. Primers used to amplify the desired PCR fragment of the genomic template DNA were 5'-CCC CTT CCC CTA AGG AAA AA-3' (forward) and 5'-ATG CTC ATC CCC AGA GAC AG-3' (reverse) (Markowski et al., 2012).

PCR-products were purified by agarose gel-electrophoresis to remove primers and dNTPs and the desired DNA-fragment/-band was extracted using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) using a QIAcube. DNA sequencing of the purified PCR-products was performed by GATC Biotech (GATC Biotech, Konstanz, Germany).

**Isolation of RNA, reverse transcription–polymerase chain reaction and quantitative real-time PCR**

Isolation of RNA from sorted cells and frozen tissue was performed using RNeasy Micro Kit® and RNeasy Mini Kit® (Qiagen), respectively, according to the manufacturer’s instructions. Reverse transcription was carried out using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). cDNA from sorted cells was preamplified using the Taqman® PreAmp Master Mix (Applied Biosystems) according to manufacturer’s protocol. Relative quantification was carried out by real-time PCR analysis using the Applied Biosystem 7500 HT Fast PCR system using commercially available gene expression assays (Applied Biosystems) and designed primer pairs listed in Table II. Quantification was performed referring to the expression of the housekeeping gene HPRT, which turned out to be expressed steadily in our experiments. Expression data with Ct-values

### Table I List of antibodies used in this study.

<table>
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<th>Antigen</th>
<th>Clone</th>
<th>Isotype</th>
<th>Dilution/concentration</th>
<th>Supplier</th>
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<td>CD24 (FACS/IHC)</td>
<td>ML5</td>
<td>PE-conjugated mouse IgG2a (FACS) unconjugated (IHC)</td>
<td>1:5 prediluted 5 µg/ml</td>
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<td>CD24 (IHC)</td>
<td>SWA11</td>
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<tr>
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<td>CD45 (FACS)</td>
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<td>FITC-conjugated mouse IgG1</td>
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<td>G155-178</td>
<td>APC-conjugated IgG2a</td>
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### Table II List of primer sets and custom assays used in this study.

<table>
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<th>Gene</th>
<th>Primer set/Taqman® assay reference number</th>
<th>Accession number</th>
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<td>Alpha SMA (ACTA2)</td>
<td>Hs00909449_m1</td>
<td>NM_00141945</td>
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<tr>
<td>CD24</td>
<td>5'-ACTGCTCCTACCCACGCACTTTA-3' (forward)</td>
<td>NM_013230</td>
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<tr>
<td></td>
<td>5'-CTTTGTTTGTTGCGATTGTGAT-3' (reverse)</td>
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<tr>
<td>β-catenin (CTNNB)</td>
<td>Hs00170025_m1</td>
<td>NM_00198209</td>
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<tr>
<td>Hypoxanthine phosphonosytransferase I (HPRT1)</td>
<td>Hs02800695_m1</td>
<td>NM_000194</td>
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<tr>
<td>5'-nucleotidase/CD73 (NT5E)</td>
<td>Hs00159686_m1</td>
<td>NM_00141945</td>
</tr>
</tbody>
</table>

from cell cultures following routine techniques as described (Klemke et al., 2009). As a rule, G-bands (GTG) were analyzed based on a resolution of at least 300 bands/haploid set. If necessary, further characterization was performed by fluorescent in situ hybridization (FISH) using three bacterial artificial chromosome clones located distal (3') and proximal (5'), respectively, of the high mobility group AT-hook (HMGA)2 locus as break-apart probe. Probe labeling was performed by nick translation either with SpectrumOrange-dUTP or SpectrumGreen-dUTP (Abbott Molecular, Wiesbaden, Germany). Fifteen microliters of the break-apart probe (concentration: 100 ng/µl) was used per slide. Co-denaturation was performed on a ThermoBrite (Abbott Molecular) for 5 min at 85°C followed by over-night hybridization in a humidified chamber at 37°C. Post-hybridization was performed at 42°C for 2 min in 0.4 x SSC/0.3% NP-40. Interphase nuclei were counterstained with DAPI (0.75 µg/ml). Slides were examined with an Axioskop 2 plus fluorescence microscope (Carl Zeiss, Gottingen, Germany); images of 100 non-overlapping nuclei were captured with a high performance CCD-camera (Visitron Systems, Puchheim, Germany) and edited with FISH View (Applied Spectral Imaging, Migdal HaEmek, Israel).
Compared to fibroids, preparations from myometrial tissue from myometrium (63% versus 19% in fibroids, Fig. 1B). The CD24hi cells are organized in clusters and have a smooth muscle phenotype. To correlate the high abundance of CD24hi cells with the pathophysiology of fibroids, the localization of CD24 within myometrial and fibroid tissue was determined by immunohistochemistry. Fibroid tissue showed a pattern of CD24 positive cells organized in clusters interspersed by unstained cells (Fig. 1C). Vice versa, CD24 positive cells were only sparsely detected in myometrium. Higher magnification showed that the stained cells had fusiform, spindle-shaped morphology. To confirm these observations, we also performed staining of fibroid and myometrial cryosectioned tissue with the antibody ML-5 which detects a formalin-sensitive epitope within the core protein of CD24 (Kristiansen et al., 2010). Regardless of some minor impacts in morphology and staining intensity caused by using cryosections, we were able to reproduce the myoma specific staining of CD24 with this antibody (Supplementary data, Fig. S2A and B). Normal tonsil tissue used as positive control showed strong staining of CD24 in the squamous epithelium layer and mantle zone of lymph follicles (Supplementary data, Fig. S2C) as reported elsewhere (Bartos et al., 1986; Sano et al., 2009). To verify a smooth muscle differentiation, double staining of smooth muscle actin (SMA) and CD24 was performed. CD24 and SMA showed apparent co-localization (Fig. 1D), indicating a smooth muscle-like differentiation of CD24 expressing cells.

CD24hi cells show a reduced proliferation rate in vivo. Because it is unclear how CD24hi cells enrich in fibroids, we looked for different proliferation by the determination of Ki-67 positive cells within the fibroid subpopulations (Fig. 2A). Ki-67 is only present in cycling cells, whereas dormant or quiescent G0 cells are negative for this marker (Scholzen and Gerdes, 2000). The overall frequency of Ki-67+ cells in fibroid cell preparations was quite low (1.6 ± 0.8%; n = 5) (data not shown) which is consistent with previous findings (Kawaguchi et al., 1991; Bourlev et al., 2003; Pavlovich et al., 2003). However, Ki-67 staining was mostly found in CD24lo cells (3.6 ± 2.9%). The CD24hi subtraction showed 14-fold lower frequency of Ki-67+ cells (0.3 ± 0.2% P < 0.05, n = 5) (Fig. 2B). This low percentage of proliferative cells indicates that CD24hi cells might reflect a quiescent subpopulation which will not enrich in tumors due to enhanced proliferation.

Additional stem cell markers are present on CD24hi cells. Based on the findings that CD24hi cells are rather quiescent and CD24 is known to be expressed on certain progenitor cells, we checked the CD24hi subsets for the presence of additional stem cell markers commonly used to identify mesenchymal stem cells (MSCs) derived from adipose tissue or bone marrow such as CD90/Thyl, CD73, CD105 and CD106 (Dominici et al., 2006; Fig. 3A). In 4/4 tumors CD24hi cells were also positive for CD73. A small proportion of CD24hi cells were also stained for CD73. CD90 was less expressed on CD24hi cells but showed strong abundance on CD24lo cells in 4/4 tumors. Expression...
of CD105 strongly varied within the subpopulations dependent on the tumor investigated. One tumor showed enhanced expression of CD105 in CD24hi cells whereas another showed elevated expression in CD24lo cells. Three additionally analyzed tumors showed no preferential expression of CD105 on either of both subpopulations. CD106/VCAM expression was low in both subpopulations. These data suggest that despite an expression of a subset of MSC markers on CD24hi cells they in general do not have a MSC phenotype.

**CD24 subpopulations give rise to smooth muscle cells and show reduced proliferation in vitro**

To check if the CD24hi subpopulation behaves differently from CD24lo cells in vitro, unsorted primary fibroid cells (Fig. 4A) were separated by FACS according to their CD24 expression, plated onto chamber slides and analyzed the next day. Phase contrast images of the subpopulations...
showed enlarged morphology of CD24\textsuperscript{hi} cells, whereas CD24\textsuperscript{lo} cells had a more compact shape (Fig. 4A, b and f). Immunocytochemical staining of CD24 confirmed the purity of the sorted cells as strong CD24 staining was detectable in CD24\textsuperscript{hi} but absent in CD24\textsuperscript{lo} sorted cells (Fig. 4A, c and g). To check if both subsets are composed of cells with smooth muscle phenotype subpopulations were seeded and stained for SMA. After the day of seeding (Day 0) CD24\textsuperscript{hi} cells showed only a moderate staining of \(\alpha\)SMA whereas CD24\textsuperscript{lo} cells exhibited a staining with stronger intensity. After cultivation for 7 days both subpopulations were entirely positive for \(\alpha\)SMA (Fig. 4B). Determination of Ki-67 index by flow cytometry after 7 days of culture revealed that the overall frequency of Ki-67-positive cells increased in both subpopulations compared with initial levels at isolation. However, the CD24\textsuperscript{hi} cells were still less positive for Ki-67 when compared with CD24\textsuperscript{lo} cells (CD24\textsuperscript{hi} 17% \(\pm\) 11 versus CD24\textsuperscript{lo} 53% \(\pm\) 16; \(n = 3, P < 0.5;\) Supplementary data, Fig. S3).

The expression of CD24 is diminished upon in vitro culture

Since there was no difference in \(\alpha\)SMA protein expression between CD24\textsuperscript{hi} and CD24\textsuperscript{lo} cells after 7 days of in vitro culture, we were interested to see if both subpopulations retain their CD24 phenotype when expanded in vitro. Sorted and mixed cells were cultured for 7 days and reanalyzed for their CD24 expression. Analysis of unsorted cells revealed that after in vitro expansion initial differentiation between CD24\textsuperscript{lo} and CD24\textsuperscript{hi} subpopulations was absent. Instead, only one population with low CD24 expression comparable with that of initially identified CD24\textsuperscript{lo} cells was observed (Fig. 5A). In accordance, sorted and 1 week cultured CD24\textsuperscript{hi} cells showed a strongly reduced expression of CD24 compared with initial mean fluorescent intensity levels and expression was nearly absent after 3 weeks of culture (data not shown). Hence, in vitro culture reduced the phenotypic difference between the subpopulations probably by influencing cellular differentiation.

The CD24\textsuperscript{hi} subfraction is detectable in xenografts

Fibroid xenografts can only be generated from freshly dissociated tumor cells, whereas in our hands cultured cells did not give rise to stable xenografts (Drosch et al., 2013). Since CD24 expression is lost upon in vitro culture it can be hypothesized that reduced expression might be correlated with a loss of tumorigenicity. To address this question, we characterized xenografts for the presence of CD24-positive cells at two time points after transplantation. Immunostaining of sectioned xenografts displayed moderate presence of CD24\textsuperscript{hi} cells 1 week post-injection (Fig. 5B and C, left). Although co-staining with Ki-67 revealed that CD24 cells were negative for this proliferation marker, a close proximity between CD24\textsuperscript{lo} and Ki-67 positive was noted (Fig. 5B). After 4 weeks of tumor growth ~50% of the cells were positive for CD24 (Fig. 5C, right) which is comparable to the frequency found in human primary fibroids.

The CD24\textsuperscript{hi} subpopulation has an immature phenotype

We further analyzed mRNA derived from both subpopulations by quantitative real-time PCR (qRT–PCR) to confirm the observed phenotypes (Fig. 6). We checked the expression levels of \(\alpha\)SMA, \(\beta\)-catenin and...
CD73. Except donor (UL5) transcript levels of αSMA were significantly higher in CD24lo cells (P < 0.005) compared with CD24hi cells confirming the previous observations made by immunocytochemistry. Control cDNA from the adenocarcinoma cell line A549 and from human fibroblasts showed no detectable expression of αSMA (data not shown). The MSC surface protein CD73 protein showed consistent but statistically insignificantly up-regulation in CD24hi cells confirming flow cytometric data. Likewise, CD24hi cells showed a moderate up-regulation of β-catenin compared with CD24lo cells which is known to be involved in stem cell regulation and smooth muscle differentiation (Willert et al., 2003; Ling et al., 2009; Giangreco et al., 2011).

**Figure 3** Mesenchymal stem cell (MSC) marker expression on CD24 subpopulations. Representative flow cytometric analysis of fibroid-derived CD31−/CD45− CD24 subpopulations displaying expression of CD73 on CD24lo cells (red graph) and CD90 on CD24hi cells (blue graphs). CD105 and CD106 were mostly indifferent between subpopulations. Isotype controls were IgG1 and IgG2a, respectively.

CD24 expression in fibroids is independent of tumor size or age but correlates with WNT4 expression in a certain genetic subgroup

Since recently fibroids have been shown to belong to different genetic subgroups showing mutually exclusive mutations in the MED12 gene or HMGA2 (Markowski et al., 2012), we were interested to analyze if CD24 expression could be correlated to one of these subgroups. qRT–PCR analysis of mRNA derived from fibroids showing either MED12 mutations (n = 16) or HMGA-rearrangements (n = 10) revealed that CD24 is highly expressed on both genetic subgroups. Besides this we also analyzed if the wingless-type MMTV integration site family, member 4 (Wnt4) which has been shown to be significantly higher expressed in fibroids with MED12 mutation (Markowski et al., 2012) also correlates with CD24 expression. A significant correlation (P < 0.05, n = 16) was observed between CD24 and Wnt4 only in tumors with mutations in the MED12 gene (Fig. 7A) including base exchanges of codon 43 and 44 which are the most frequent MED12 mutations in fibroids (Mäkinen et al., 2011). In turn, fibroids with 12q14–15 rearrangements (Fig. 7B) did not exhibit this correlation between the expression of CD24 and Wnt4 in n = 8 tumors (WNT4 signal was not detectable in 2/10 tumors). CD24 expression did not correlate with tumor size or patient age in tumors in both groups (data not shown; please note also Supplementary data, Table SI for further details).

**Discussion**

Based on the identification of SP cells in fibroid and myometrial tissue, myomagenesis can be considered as the result of dysregulated stem/
progenitor cells (Ono et al., 2007, 2012; Mas et al., 2012). Our study strongly substantiates the concept of a ‘dysregulated progenitor hypothesis’ by the identification of a certain cellular subpopulation in uterine fibroids characterized by the abundant expression of the surface marker CD24. We were able to show that this surface protein is strongly expressed on a subset of cells in fibroid tissue. Although CD24 cannot be considered being an unequivocal stem cell marker (Al-Hajj et al., 2003; von Furstenberg et al., 2011), this protein tends to be mostly expressed on undifferentiated or progenitor cells or tumor-initiating cells in different malignancies (Lim 2005; Fang et al., 2010; Lee et al., 2011). As uterine fibroids are considered to arise from a single cell clone (Holdsworth-Carson et al., 2013), it is tempting to speculate that CD24hi cells are derived from a transformed and mutated myometrial stem/progenitor cells possibly being also positive for CD24. We could not provide direct evidence for enhanced self-renewal or in vivo tumor-regeneration which are considered to be main features of stem cells (Gonzales and Bernad, 2012) because this would be dependent on the cells’ ability to be propagated extensively in vitro. This is hampered by the very short in vitro lifespan of MED12-mutated cells (Markowski et al., 2014) and an almost subsequent activation of senescence pathways upon culture (Markowski et al., 2010) as well as the inability of fibroid cells to grow in soft-agar (Drosch et al., 2013). Nevertheless, CD24hi cells exhibit certain features also observed in progenitor cells. Akin to stem cell-like SP cells isolated from myometrium and fibroids (Ono et al., 2007, 2013) CD24hi cells showed a significantly reduced expression of differentiation markers such as αSMA and a quiescent G0 state as indicated by the absence of Ki-67 in CD24hi cells. Interestingly, xenografts generated from primary fibroid cells showed a high proportion of CD24hi cells. Although these cells were negative for Ki-67 they were still detectable after prolonged tumor growth suggesting that CD24hi cells persist as immature progenitor-like cells within the tumors. This assumption is further supported by the reduced expression of CD90 which is believed to be a marker of terminally differentiated fibroid cells (Chang et al., 2010). In turn, strong expression of the MSC marker CD73 (Dominici et al., 2006) was revealed in CD24hi cells using both flow cytometry and qRT-PCR. As this is an additional feature shared with the SP cells isolated by the Hoechst efflux method (Mas et al., 2012), we consider a certain overlap between both phenotypes. However, the high abundance of the CD24hi population in fibroid tissue in comparison to the fibroid SP makes it more likely that those cells could reflect some intermediate progenitor cells rather than infrequent early stem cells. In contrast to freshly isolated cells, in vitro cultured CD24hi cells showed a phenotypic change with increased αSMA staining and reduced expression of CD24. This is in accordance with observations made by several other groups describing loss of hormone receptors (Severino et al., 1996; Gargett et al., 2002) or massive changes in gene expression profile also confirming a significant down-regulation of CD24 in cultured fibroid cells (Zaitseva et al., 2006). Interestingly, the authors from the previously study by Chang et al. (2010) observed that less differentiated CD90− fibroid cells (which we found out to be CD24hi) shift to terminally differentiated CD90+ cells following in vitro culture. In general, not only differentiation/dedifferentiation upon culture but also a selection against MED12 mutated cells (Markowski et al., 2014) may account for some of the above observations. Hence, it seems to be conceivable that similarly CD24hi cells could be replaced by differentiated CD24lo cells. Based on this assumption, it can be hypothesized that loss of CD24 expression might also correlate with the loss of in vivo growth.

Figure 4 Phenotype of sorted subpopulations. (A) (a) Crude preparation of unsorted cells labeled with CD24 (ML5) and Alexa555-conjugated secondary antibody (orange). (b) After 1 day cultured CD24hi cells showed an elongated morphology while (f) CD24lo cells showed a more compact shape. (c and g) Staining with CD24 confirmed purity of sorted cells. (B) Expression of αSMA (red) in CD24 subpopulations 1 day post-seeding (Day 0) and after 7 days of in vitro culture (red). Nuclei were counterstained with DAPI.
Figure 5 Effects of in vitro culture on CD24 expression. (A) Both sorted and unsorted primary cells were cultured and reanalyzed after 1 week. Top: Unsorted cells showed a strong reduction of CD24 after in vitro culture (dotted line) compared with initial protein expression (bold line). Bottom: Sorted CD24\(^{hi}\) and CD24\(^{lo}\) cells showed down-regulation of CD24 after 1 week of in vitro culture. (B) Histological analysis of xenografts generated by injection of uncultured, unsorted cells. Staining of CD24 (orange) and Ki-67 (green) in cryosections after 1 week post-injection displayed absence of proliferation marker in CD24\(^{hi}\) cells but close proximity between both cell types (scale bar 100 \(\mu\)m). (C) Staining of CD24 in xenografts after 1 week (left) and 4 weeks (right) of growth showing high abundance of CD24\(^{hi}\) within xenograft. Scale bars 500 \(\mu\)m and 50 \(\mu\)m. Note isotype-specific control in insert lower left were only counterstain of xenografts tissue is visible.
capacity of fibroid cells. Indeed, cultured—and therefore CD24lo cells—are unable to form stable xenografts (Drosch et al., 2013). Myometrial cells, which we determined to be mostly CD24lo, are also unable to form tumors after injection (Suo et al., 2009; Drosch et al., 2013). Vice versa, injections of uncultured cells produced xenografts with a pronounced proportion of CD24hi/Ki-67+ cells. These resting cells were often located in proximity to CD24lo/Ki-67+ cells. As Szotek et al. (2007) found non-proliferative label-retaining cells adjacent to dimmer c-Kit positive cells they proposed a direct lineage of c-Kit positive cells from the rather quiescent LRCs. Likewise, it can be speculated that CD24lo/Ki-67+ cells might be also derived from the non-proliferative CD24hi cells. This theory is supported by the observation that normal myometrium displays a low abundance of CD24hi cells, thus reflecting the physiological state. This mature tissue made up of differentiated cells does not exhibit lots of changes apart from myometrial hyperplasia during pregnancy (Shynlova et al., 2010). Hence, the infrequent CD24hi cells in myometrium might represent some kind of immature reserve cells which could give rise to the CD24hi mature myometrial cells upon activation. As recently a paracrine activation of the WNT-pathway has been shown between mature and SP cells in fibroids (Ono et al., 2013), it can be speculated that also a paracrine signaling between the CD24 subpopulations regulate differentiation processes between both cell types. Interestingly, we observed that CD24hi cells tend to show an enhanced expression of beta-catenin which could also reflect the effects of paracrine signaling. Although we could not provide a direct evidence for a true stem cell phenotype of CD24hi cells we are convinced that CD24hi cells are a crucial tumorigenic factor. However, it still remains an absorbing question how CD24hi cells may enrich in fibroid tissue. As CD24hi exhibit a low Ki-67 index, it is rather unlikely that these cells will enrich due to hyperproliferation. A possible scenario for enrichment could be that an altered, potentially blocked differentiation towards CD24lo cells leads to accumulation of CD24hi cells. This hypothesis is supported by a recently identified mutation present in almost 70% of all fibroids (Mäkinen et al., 2011), i.e. affecting the gene encoding for the Mediator complex subunit 12 (MED12). MED12 is considered to be involved in regulating stem cell function and embryonic development (Tutter et al., 2009). Fibroids carrying MED12 mutations show alterations in the canonical WNT-signaling pathway (Mäkinen et al., 2011) which in turn facilitates stem cell differentiation and self-renewal via downstream targets such as beta-catenin (Clevers, 2006). Beta-catenin itself is known to cause fibroid-like lesions after uterus-specific overexpression in a mouse model (Arango et al., 2005;
Tanwar et al., 2009). Intriguingly, we found a significantly positive correlation between the expression of CD24 and one of the WNT-pathway members, WNT4, in tumors with MED12 mutation whereas tumors with HMGA2-rearrangements did not exhibit any correlation. As we also observed that CD24hi cells tend to show a higher expression of β-catenin. Besides an involvement in paracrine mechanisms (Ono et al., 2013) this might also lead to potential explanations for the enrichment of CD24hi cells and myogenesis: Mutations, e.g. in MED12 occurring in a precursor cell may lead to impaired beta-catenin/wnt-signaling in CD24hi cells resulting in impaired differentiation and accumulation of CD24hi cells. This might lead to a reduced differentiation of CD24hi into CD24lo cells and result in a clonal accumulation of CD24hi cells in fibroids which is in concert with the findings about the clonality of fibroids cells (Holdsworth-Carson et al., 2013). Since not only WNT-signaling exerts pleiotropic functions dependent on cellular context and relative amount of ligand (as reviewed in Ling et al., 2009) further efforts should be made in studying the pivotal functions of this pathway during myogenesis. Pathway and cytogenetic analyses will contribute to better understanding of the functional link between ‘fibroid-mutations’, wnt-pathway, CD24 and stem cell functions in human fibroids and myometrium. Taken together, we identified a subpopulation characteristic for uterine fibroids. This CD24hi subpopulation shows certain properties also common in progenitor cells. These cells might enrich in fibroid tissue due to altered differentiation and erroneous control mechanisms. The further characterization of this fibroid subpopulation and its origin will contribute to a better understanding of fibroid pathophysiology and might open up possibilities for the development of new treatment strategies.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgements
The authors thank Prof. Bernd Bojah, Klinik für minimalinvasive Chirurgie, Priv.-Doz. Dr. Herbert Mecke Klinikum Auguste-Victoria and P.D. Dr Silvia Mechsner, Charité Klinikum Benjamin-Franklin for providing them with fibroid and myometrial tissue as well as Nadja Bergmann, Simone Thalheim, Frauke Meyer and Robert Jacobi for excellent technical assistance. They also acknowledge the help of Prof. Altevogt from the German Cancer Research Centre who kindly provided them with the CD24 antibody.

Funding
No external funding was either sought or obtained for this study.

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