

Overexpression of the truncated form of High Mobility Group A proteins (HMGA2) in human myometrial cells induces leiomyoma-like tissue formation

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ABSTRACT: The pathogenesis of uterine leiomyomas, the most common benign tumor in women, is still unknown. This lack of basic knowledge limits the development of novel non-invasive therapies. Our group has previously demonstrated that leiomyoma side population (SP) cells are present in tumor lesions and act like putative tumor-initiating stem cells in human leiomyoma. Moreover, accumulated evidence demonstrates that these benign tumors of mesenchymal origin are characterized by rearrangements of the High Mobility Group A proteins (HMGA). In this work, we tested the hypothesis that leiomyoma development may be due to overexpression of HMGA2 (encoding high mobility group AT-hook2) in myometrial stem cells using *in vitro* and *in vivo* approaches. Our work demonstrates that the truncated/short form of HMGA2 induces myometrial cell transformation toward putative tumor-initiating leiomyoma cells and opens up new possibilities to understand the origin of leiomyomas and the development of new therapeutic approaches.

Key words: High Mobility Group A proteins / side population / human myometrium / uterine leiomyomas / somatic stem cells

Introduction

Uterine fibroids or leiomyomas represent the most common benign tumors of the female reproductive tract. Although severe symptoms only develop in 15–30% of Caucasian and African-American women, around 70–80% women worldwide have at least one fibroid (Baird *et al.*, 2003; Bulun, 2013).

These tumors are characterized by their well-defined encapsulated fascicular formation inside the myometrium. Histologically, they are constituted by bundles of smooth muscle cells with high extracellular matrix (ECM) content of collagen, fibronectin and proteoglycans, which contribute for a substantial portion of tumor volume. A remarkable feature of uterine leiomyomas is their dependency on the ovarian steroids estrogen and progesterone (Ishikawa *et al.*, 2010). They can affect women's health by causing heavy, irregular or prolonged menstrual

bleeding (Stewart, 2001; Parker, 2005). Also they have been associated with subfertility, recurrent abortion and preterm labor creating an important economic impact on healthcare systems worldwide (Flynn *et al.*, 2006; Parker, 2007; Catherino *et al.*, 2011). However, despite their high prevalence, the etiology of fibroids is poorly understood and their standard treatment is still surgical removal.

In recent years, two main hypotheses have been explored to understand the origin of fibroids: the cellular (Bulun, 2013) and/or the chromosomal/genetic origin (Hammond and Sharpless, 2008; Cha *et al.*, 2011; Markowski *et al.*, 2011). Therefore, considering that uterine leiomyomas are monoclonal tumors (Linder and Gartler, 1965) it is possible that the dysregulation of myometrial somatic stem cells (SSCs) or committed cells that acquire stem-like features could be responsible of this benign condition. Based on these premises, two independent groups demonstrated the existence of putative SSCs with the characteristics of

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tumor-initiating cells (TICs) in uterine leiomyomas (Mas *et al.*, 2012; Ono *et al.*, 2012). A variety of somatic chromosomal abnormalities has been described in 40–50% of uterine leiomyomas, such as deletions of 7q and rearrangements involving 12q14–q15 or 6p21 (Sandberg, 2005; Hodge *et al.*, 2012). Additionally, whole-genome sequencing showed that these chromosomal rearrangements are often complex, consisting of multiple chromosomal breaks and random reassembly (Mehine *et al.*, 2013).

TICs carry mediator complex subunit 12 (MED12) mutations, suggesting one genetic mutation interacts with the stem cell that might transform myometrial toward leiomyoma cells (Ono *et al.*, 2012) bridging the gap between both hypotheses.

High Mobility Group A (HMGA) genes encode DNA-binding non-histone proteins that control cell growth by indirect regulation of the DNA transcription process. They control cell-cycle checkpoints and repair of DNA double-strand breaks (Mehine *et al.*, 2013). As expected, their expression is high during embryogenesis (Li *et al.*, 2006), whereas it remains undetectable in differentiated adult tissues (Rogalla *et al.*, 1996) and could be regulated in response to growth factors. HMGA2 is the driver gene for tumors carrying 12q14–q15 rearrangements (Fusco and Fedele, 2007; Mehine *et al.*, 2013). In leiomyomas, chromosomal band 14q24 is the HMGA2-targeted translocation partner (Ligon and Morton, 2000; Mehine *et al.*, 2013) suggesting that overexpression of HMGA2 may be a key event in the pathogenesis of leiomyoma with chromosomal rearrangements affecting the region 12q14.3 (Medeiros *et al.*, 2007), as well as in those without chromosomal aberrations (Klemke *et al.*, 2009).

In this work, we aim to demonstrate that overexpression of truncated/short HMGA2 form in myometrial cells would result in an abnormal proliferation of the SSC niche leading to the formation of leiomyoma-like tissue.

Materials and Methods

Tissue samples

Samples of human leiomyoma and human myometrium used in this study were obtained from Caucasian patients undergoing laparoscopic myomectomy for symptomatic uterine fibroids, or hysterectomy for uterine prolapse. The use of human tissue specimens was approved by the Institutional Review Board of Instituto Valenciano de Infertilidad (registered under number 1006-C-072-CS-F) and signed informed consent was obtained.

Tissue preparation and SP isolation

Leiomyoma fragments were carefully dissected out, minced manually into small pieces (1–2 mm³) and digested at 37°C by enzymatic means to obtain leiomyoma cell suspension (LeioTF; Mas *et al.*, 2012). LeioTF were stained with Hoechst 33342 dye (Ho-33342; Sigma-Aldrich) and subjected to a flow-cytometry analysis to separate the human leiomyoma side population (LeioSP), to the non-side population (LeioNSP) cells according to our previously published protocols (Cervelló *et al.*, 2010). Based on these selection criteria, two human leiomyoma SP cell lines LeioSP1 and LeioSP2, were generated by our group previously (Mas *et al.*, 2012) and used for further molecular experiments in this work. Additionally, primary human myometrial cells (hMyo) were isolated from patients undergoing hysterectomy for uterine prolapse and they were used as controls of molecular experiments. Some of these hMyo cells were also stained with Hoechst 33342 dye (Ho-33342; Sigma-Aldrich) and subjected to a flow-cytometry analysis to separate the human myometrial side population (MyoSP) for further transfection assays.

RNA extraction and PCR

Total RNA extraction was performed by TRIzol method according to the manufacturer's recommended protocol (Life Technologies, NY, USA). Primers used for PCR amplification were (HMGA2-full/long; exons 1–5) Fw-5'-CCAGCGCCTCAGAAGAGAGGA-3' and Rv-5' CTTCGGCAGA CTCTTGTA-GGA-3' and (HMGA2-truncated/short; exons 1–3) Fw-5'-GCCGTCCACTTCAGCCCAGG-3' and Rv-5' CCTAGGTCTGCCTCTT GGCCGT-3' (Fig. 1). Our positive control for HMGA2 expression was the human embryonic stem cell (hESC) line VAL-5 (Aguilar-Gallardo *et al.*, 2010). Distilled water was used as a negative control in all cases. Moreover, GAPDH was used as an internal control in all the gene expression studies. Finally, all the bands obtained corresponding to candidate mRNA amplifications were isolated and sequenced to verify the identity of the PCR products.

Plasmid constructs

Total RNA was isolated from a hESC line, VAL-5 cell (Aguilar-Gallardo *et al.*, 2010) with Trizol reagent. First-strand cDNA was synthesized from 1 µg of total RNA using random hexamers and expand reverse transcriptase (Roche Diagnostics, Barcelona, Spain). The cDNA was used as a template for conventional PCR using specific primers for the determination of

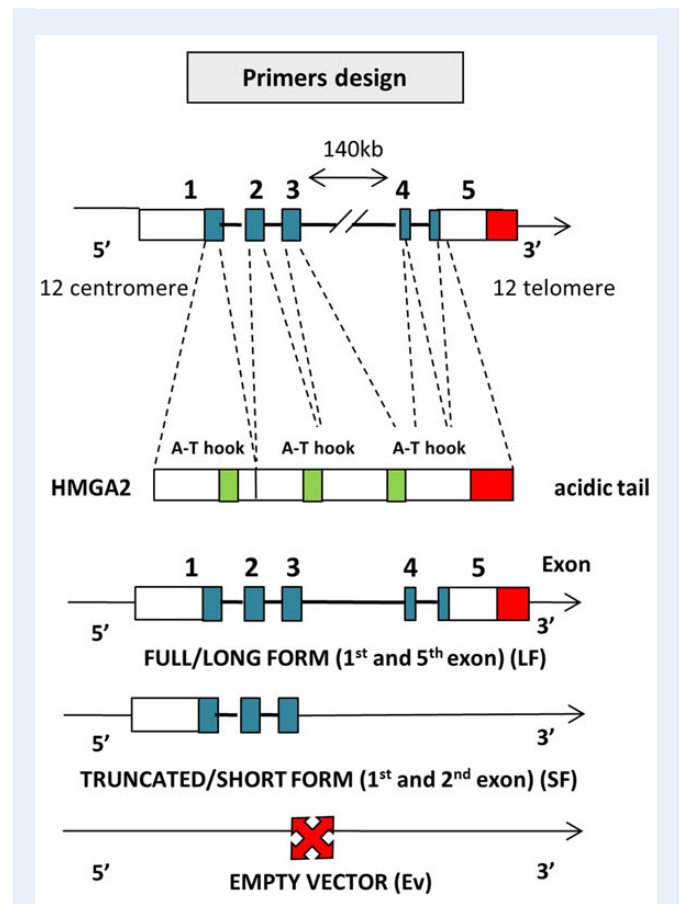


Figure 1 Overexpression of High Mobility Group A proteins (HMGA2) in leiomyoma SP derived cells. (**UPPER PANEL**) Schematic representation of the human HMGA2 gene and protein and primers design for different isoforms of HMGA2 (see primers in Materials and Methods). (**LOWER PANEL**) Plasmid construct used in this study, carrying the cDNA for full or long form (LF), truncated or short form (SF) and empty vector (negative control) form (Ev) of HMGA2, respectively.

HMGA2 isoforms (sequences available on demand). A 330 bp (HMGA2-full/long; exons 1–5) or 249 bp (HMGA2-truncated/short; exons 1–3) fragments containing the complete cDNA coding sequence were cloned into the EcoRI/XhoI restriction sites of the pCAGEN vector (Addgene plasmid 11160) kindly provided by Dr Matsuda (Howard Hughes Medical Institute, Boston, USA) (Matsuda and Cepko, 2004). All constructs were confirmed by nucleotide sequencing (Supplementary data, Fig. S1).

Cell culture conditions and transfection experiments

Primary human myometrial cells (hMyo), myometrial Side Population cells (MyoSP) and a human uterine smooth muscle cell line (hUtSMC) (Promocell, C12575, Germany) were grown in supplemented medium in a hypoxic environment (2% O₂, 37°C, 5% CO₂, 90% humidity) (Cervelló et al., 2011; Mas et al., 2012). For transfection experiments, Lipofectamine™2000 Transfection Reagent (Life Technologies) was used. For each transfection condition, samples were diluted with 4.0 µg of DNA from different HMGA2 isoforms (full/long protein, truncated/short protein as well as empty vector as a negative control) in 10 µl of Opti-MEM® I Reduced Serum Medium.

Real-time PCR for myometrial HMGA2 transfected cells

Total RNA from different myometrial HMGA2 transfected cells (hMyo, hUtSMC and MyoSP) was extracted using TRIzol method. Quantitative real-time PCR was performed in triplicate using Light Cycler FastStart DNA Master SYBR green I (Roche) in a Light Cycler 480 (Roche). hESC line VAL-5 (Aguilar-Gallardo et al., 2010) RNA was used as positive control to test the expression of HMGA. Distilled water was used as a negative control in all cases. Finally, mRNA level was normalized to GAPDH as a housekeeping gene. The primers sequences used were (HMGA2-full/long; exons 1–5) Fw-5'-GCGGCCGAATTCATGAGCGCACGCG-GTGAGGGCG-3' and Rv-5'-GCGGCCCTCGAGCTAGTCCTCTTCGGC and (HMGA2-truncated/short; exons 1–3) Fw-5'-GCGGCCGAATTCATGAGCGCACGCGGTG-AGGGCG-3' and Rv-5'-GCGGCCCTCGAGCTACCATTTCCTAGG-3'.

Clonogenicity assay

Human myometrial cells (hMyo, hUtSMC and MyoSP) transfected with HMGA2 isoforms (HMGA2-truncated/short, HMGA2-full/long or HMGA2-empty vector) were seeded in 6-well tissue culture plates at 156 cells/cm² clonal density ($n = 3$ for each condition) in a hypoxic environment (Chan et al., 2004). After 15 days, cloning efficiency (CE) was determined from the CE formula (%) = (number of colonies/number of cells seeded) × 100. The mean and SEM were obtained from each clonal density and were calculated for each cell type. Statistical analysis of the data was performed with SPSS software and Student's *t*-test was used to evaluate their significance. *P*-value of <0.05 was considered statistically significant.

Soft agar assay

Soft agar assay was performed according to the technique described by Fedele et al. (1998). In brief, 40 × 10⁴ myometrial HMGA2 transfected cells were suspended in 0.5 v/v agar mixed with supplemented medium in 60 mm dishes for 15–30 days. After this period, colonies larger than 64 cells were evaluated microscopically, counted and their area measured. Assays were conducted in triplicate. The mean and SEM obtained from each cell area was calculated in each cell type (hMyo, hUtSMC and MyoSP). Statistical analysis of the data was performed by a Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

Molday Ion Rhodamine staining and flow-cytometry analysis

A method using fluorescent iron-oxide nanoparticles was chosen to label myometrial HMGA2 transfected cells (hMyo, hUtSMC and MyoSP) in order to track them *in vivo* in the animal model. The method called 'Molday Ion Rhodamine B' (CL-50Q02-6A-50, Biopal) consists of superparamagnetic iron-oxide contrast reagent (SPIO) labeled with rhodamine B. This property allowed labeled cells to be sorted by flow cytometry (between 555 and 565–620 nm) and detected by Prussian Blue staining in paraffin embedded xenograft tissue due to the iron deposits. Molday Ion Rhodamine B was added at 25 µg/ml and incubated for 24 h. Then, cells were visualized by fluorescent Nikon Eclipse microscope and resuspended in phosphate buffered saline for flow-cytometry analysis in order to determine the percentage of stained cells.

Xenotransplantation assays

All animal experiments were performed in female non-obese diabetic mice, NOD-SCID (strain code394; NOD.CB17-Prkdc^{scid}/NcrCrI, Charles River Laboratories) and conducted according with the Institutional Review Board and the Ethics Committee of the Fundación Instituto Valenciano de Infertilidad, Instituto Universitario IVI-University of Valencia, INCLIVA, Valencia, Spain. Mice were ovariectomized to eliminate the influence of endogenous estrogen (E₂) and progesterone (P₄) (Cervelló et al., 2010; Mas et al., 2012). Two different methods based on different injection sites (kidney capsule or uterine horn) (Ono et al., 2007; Ishikawa et al., 2010) were used with different HMGA2 constructs, in a total of 24 treated animals. As the first approach, 12 animals were injected beneath the renal capsule with single-cell suspensions of hMyo, hUtSMC and MyoSP (from 500 000 to 1 000 000 cells) transfected with the different isoforms of HMGA2. Moreover, three more animals were injected with non-transfected myometrial cells and used as a negative control.

After assessing the results from our first experiment, we performed the injection of hMyo cells transfected with HMGA2-truncated/short and HMGA2-empty vector (control) isoforms or hMyo non-transfected cells, into the right (empty vector HMGA2 construct cells/non-transfected cells) and left (truncated/short HMGA2 construct cells) uterine horns, respectively, in four animals. We repeat the same procedure in hUtSMC transfected with HMGA2-truncated/short and HMGA2-empty vector (control) isoforms or hUtSMC non-transfected cells into each uterine horn in four animals. Finally, MyoSP cells transfected with HMGA2-truncated/short and HMGA2-empty vector (control) isoforms and MyoSP non-transfected cells were injected into each uterine horn in four animals. In this case, we discarded HMGA2-full/long form due to inconsistent tumor growth pattern obtained from the first experiment (see discussion).

In all cases, NOD-SCID mice were implanted subcutaneously with E₂ pellets at the neck level (SE121, 17β-estradiol 0.18 mg/60 days; Innovative Research of America) combined with 1 mg of subcutaneous progesterone (P₄, Dr Carreras, Hospital14, Barcelona, Spain) for 2 weeks within a 3-week free interval. Finally, all xenotransplanted mice were maintained in specified pathogen-free facilities and fed *ad libitum* until they were sacrificed, according to the experimental protocol for a total of 60 days after the cell implantation.

Immunohistochemical validation of human leiomyoma-like tissue formation in xenotransplants

Hematoxylin and eosin (H&E; Bancroft and Stevens, 1996) staining was used for standard visualization. We performed Prussian blue staining in order to visualize the presence of iron deposits found inside cells treated with Molday ION. Murine spleen and murine lung tissues were used as positive

and negative controls, respectively, for this procedure. Alcian Blue pH 2.5 staining (Merck) was also used to detect ECM components in the leiomyoma-like tissue generated, since this dye has affinity for carboxylated acid glycoproteins and mucopolysaccharides. The positive and negative controls used in this assay were small intestine and myometrium, respectively (Mas et al., 2012).

To further assess the proliferative activity of normal and tumor tissue in both reconstructed xenografts models, we performed immunohistochemical analysis for the proliferation-related antigen Ki-67. Sections were incubated in a prediluted ready-to-use rabbit monoclonal antibody (IgG) (CONFIRM anti-Ki-67, 30-9 clone, Roche-Ventana) on an automatized immunostainer (Benchmark XT-Ventana Medical Systems) using the Ventana/VIEW DAB detection kit following the instructions of the provider.

Finally, immunohistochemistry analyses were done to demonstrate the human origin of cells by using a human specific anti-progesterone receptor (hPR (EP1516Y, Abcam)) in both reconstructed xenografts models.

Immunolocalization of the specific cell populations in all the samples analyzed was visualized and photographed using a Nikon Eclipse 80i camera attached to the microscope.

Results

Human leiomyoma cells expressed the truncated/short HMGA2 form

Human leiomyoma cells (LeioSP, LeioTF, LeioSPI and LeioSP2), presented an enriched expression pattern of the truncated/short HMGA2 compared with human myometrial (hMyo) cells (Fig. 2A and B).

Overexpression of the full/long form (LF) or truncated/short form (SF) of HMGA2 constructs were checked in our transfected myometrial cells: hMyo (Fig. 2C), hUtSMC (Fig. 2D) and MyoSP (Fig. 2E). Forty-eight hours after transfection either with an empty vector (Ev) or with the vector containing LF or SF. Untransfected hMyo, hUtSMC and MyoSP cells were used as negative controls to test the exogenous expression of our gene of interest. RT-PCR experiments revealed a robust increase of SF and LF isoforms in all myometrial cells transfected, whereas in both empty vector (Ev) and untransfected cells these molecules were similarly expressed (Fig. 2C–E).

Phenotype and clonogenic activity of HMGA2 transfected human myometrial cells

To visualize the phenotype of these stable transfectants, we analyzed their ability to grow in soft agar (Fig. 3A). Results obtained significantly support a trend to an increased proliferation ability of all myometrial cell types (hMyo, hUtSMC and MyoSP) transfected with HMGA2 (SF) that form larger growing colonies in soft agar compared with full/long HMGA2 (LF) or empty vector (Ev) (P -value ≤ 0.05 ; Fig. 3C, E and G). Interestingly, non-transfected cells did not form colonies after 15–30 days, indicating no anchorage-independent growth, and was excluded for the final analysis.

On the basis of the clonogenic efficiency (CE) assay, several investigators have suggested the presumed clonal origin of uterine leiomyoma from myometrial cells (Walker and Stewart, 2005). We decided to culture *in vitro* HMGA2 transfected myometrial cells in hypoxic culture under 2% oxygen tension (Cervelló et al., 2010, 2011) and tested the CE of hMyo, hUtSMC and MyoSP transfected with HMGA2 isoforms (HMGA2-truncated/short, HMGA2-full/long and HMGA2-empty vector) (Fig. 3B, D and F). Results confirmed that clonogenicity was

significantly higher in hMyo cells transfected with HMGA2-truncated/short construct (hMyo SF) compared with other isoforms (hMyo LF and hMyo Ev) (P -value ≤ 0.05 ; Fig. 3B). Also, hUtSMC and MyoSP transfected with the SF construct showed higher CE compared with other isoforms; however, the differences were not statistically significant (Fig. 3D and F).

Reconstruction of human leiomyoma-like tissue from hMyo and MyoSP cells with truncated/short HMGA2 isoform in an animal model

Before injection, myometrial HMGA2 transfected cells (hMyo, hUtSMC and MyoSP) were analyzed by flow cytometry to detect the rhodamine fluorescence signal derived from the SPIO staining for tracking purposes. In all cases, the percentage of living cells with rhodamine B positive signal at 575 nm was around 85–95% (data not shown), thus revealing that labeling with SPIO did not significantly affect cell viability.

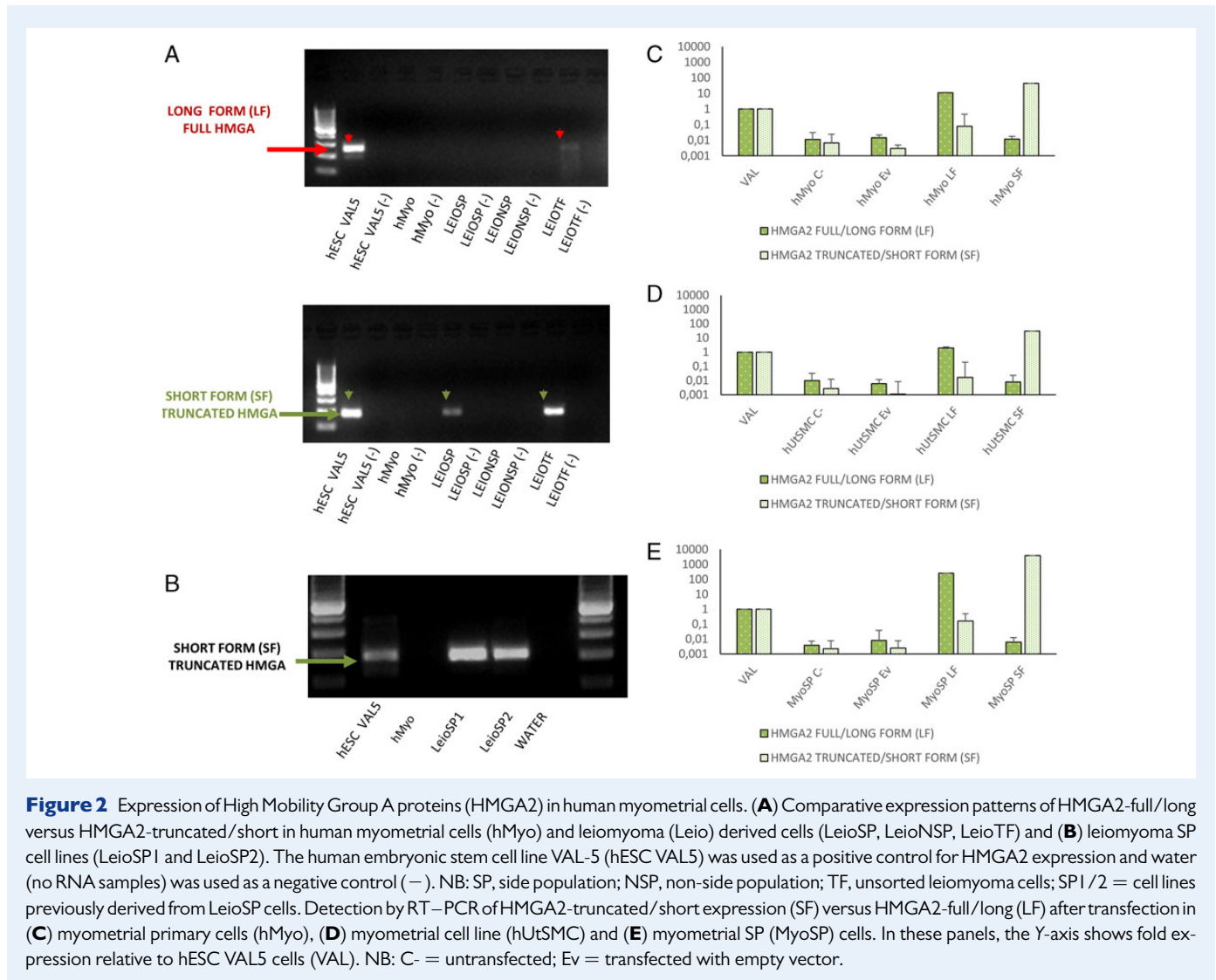
After injection, animals were maintained for 8-weeks with E2/P4 supplement then sacrificed and their kidney capsule ($n = 12$) or the uterine horns ($n = 12$) removed and carefully analyzed. In the kidney capsule model, hMyo and MyoSP transfected cells had the ability to form tumors (Supplementary data, Table S1). Indeed, these tumors were macroscopically (Fig. 4A) and histologically (Fig. 4B) similar to the original fibroid tissue. hUtSMC cells transfected with truncated/short HMGA2 form were able to grow as cell graft; however, the tumor volume was not as significant as in the other myometrial cell types (hMyo and MyoSP) (Supplementary data, Table S1).

The presence of human cells in these tumors was confirmed by Prussian blue dye (Fig. 4C). The Ki67 staining in xenograft tissues demonstrates a moderate proliferative pattern in hMyo and MyoSP truncated/short HMGA2 transfected cells (Fig. 4D). We further confirmed the leiomyoma-like composition by Alcian Blue staining, detecting ECM components like glycoproteins and carboxylated acid mucopolysaccharides (Fig. 4E). Finally, human progesterone receptor (hPR) staining was demonstrated in growing tissue in the murine kidney capsule, confirming the human origin of the leiomyoma-like tissue (Fig. 4F).

The rate of success observed in the intrauterine injection model with hMyo and MyoSP transfected with the truncated/short HMGA2 form was higher than in the kidney capsule and hUtSMC cells transfected with truncated/short HMGA2 form (Supplementary data, Table S1). Cell-derived xenografts mimic uterine leiomyoma shape at macroscopic and histological appearance (Fig. 4G and H). To confirm the reconstruction of human leiomyoma-like tissue at the defined injection site, positive results were observed by the presence of Prussian blue dye (Fig. 4I). Moreover, the positive Ki67 staining (Fig. 4J) with the co-expression of Alcian Blue (Fig. 4K) and hPR (Fig. 4L) further confirmed the nature of the human myometrial transfected cells to reconstruct leiomyoma-like tissue in an animal model.

Discussion

The expression of HMGA2 is widely restricted to the embryonic stage and decreases to undetectable levels in adult tissues. HMGA2 was characterized by important functions related to cellular growth and differentiation in the development process. Disturbed expression in adult tissues has an important role in the growth of several mesenchymal tumors due to its oncogenic ability (Schoenmakers et al., 1995; Bulun, 2013).



Uterine leiomyomas are benign tumors composed by disordered smooth muscle cells arising from the human myometrium. This tumorigenic phenomenon occurs when tissue-specific cells suffer chromosomal aberrations, genetic alterations or epigenetic modifications (Bulun, 2013). Rearrangements of HMGA genes and thus alterations of their related proteins have been associated with aberrant mesenchymal growth manifested in other benign tumors, such lipomas (Schoenmakers et al., 1995) or endometrial polyps (Dal et al., 1998). Thus, some of these genetic alterations could be related to the gene that encodes to HMGA2 and probably give rise to a subset of uterine leiomyomas (Schoenmakers et al., 1995; Hennig et al., 1999). Additionally, in 2006 an elegant study in a mice model demonstrated that chromosomal translocations of HMGA2 provoke repression or activation of the different isoforms of this gene causing benign mesenchymal tumors. This aberrant HMGA2 overexpression could be detected in the pool of mesenchymal differentiated cells and it is associated with the tumorigenic potential independently of the nature of the HMGA2 transcripts (Zaidi et al., 2006).

MED12 mutations are specific and high frequency genetic events in uterine leiomyomas (Makinen et al., 2011). However, a recent publication demonstrates that MED12 and HMGA2 mutations are two

independent genetic events in the tumorigenesis of uterine leiomyomas, suggesting that truncated/short HMGA2 *per se* could be the key mechanism by which the chromosomal rearrangements contribute to human tumor development (Bertsch et al., 2014).

Our results also support the cell theory suggesting that uterine leiomyomas arise clonally from the proliferation of smooth muscle cells or their progenitors (Walker and Stewart, 2005). Based on these premises, recently two groups demonstrated the existence of SP cells with the characteristics of TICs in uterine leiomyomas which are likely involved in leiomyoma tumorigenesis (Mas et al., 2012; Ono et al., 2012). Also, the role of HMGA2 gene in self-renewal ability in the stem cell niche and its relation to tumor growth has been reported (Nishino et al., 2008; Markowski et al., 2011) suggesting that this genetic target could transform the myometrial stem cell.

In this work, we take one step forward by examining the transforming ability of induced rearranged forms of HMGA2 (truncated/short and full/long) in several human myometrial cell types (hMyo, hUtSMC and MyoSP). We aim to investigate whether the loss of the spacer and acidic domains is sufficient to confer tumorigenic features to the HMGA2 as some researchers have previously proposed (Fedele et al., 1998;

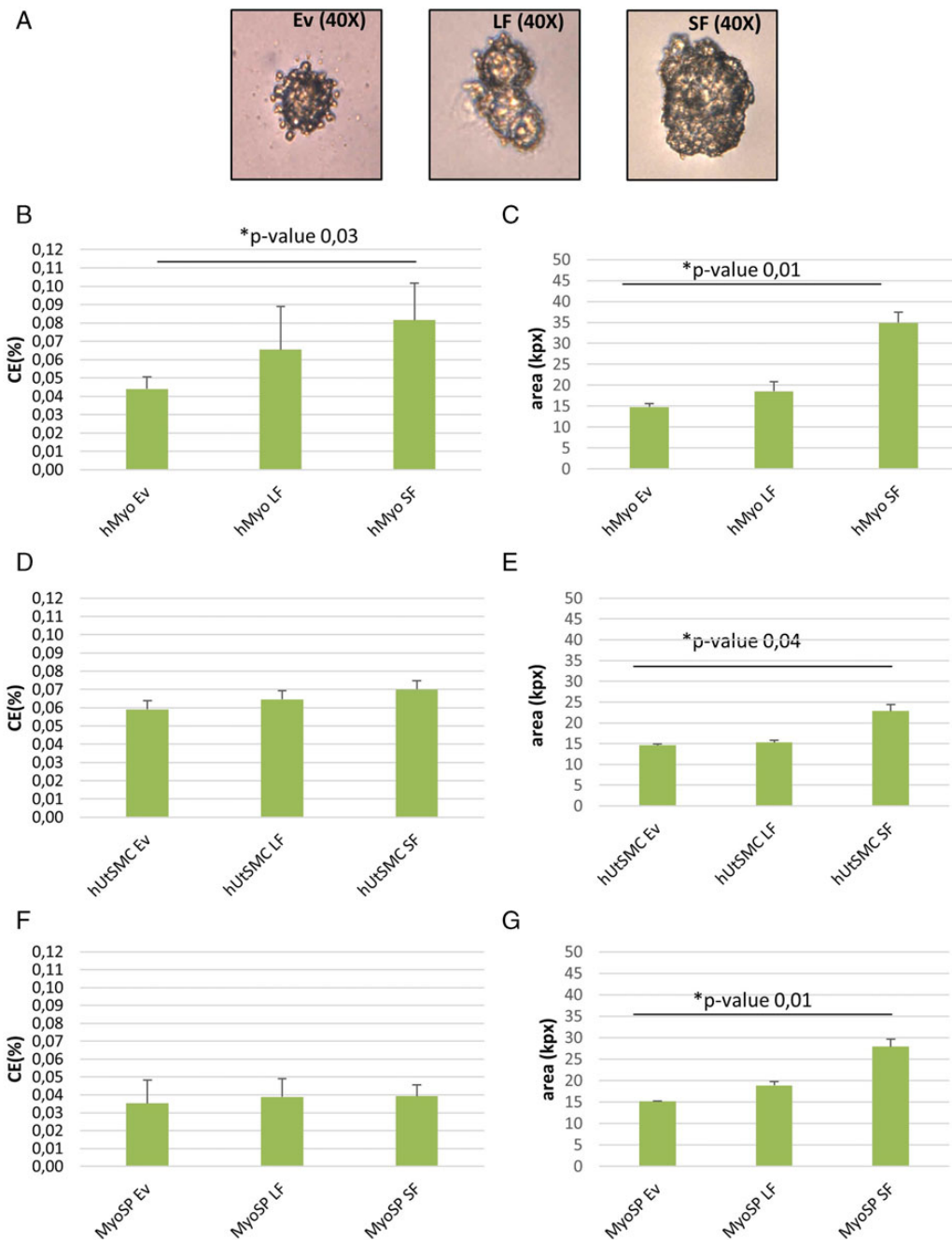


Figure 3 Growth properties of cells transfected with different High Mobility Group A proteins (HMGA2) vectors. **(A)** Images comparing colonies transformed with empty vector (Ev; control) or the long (LF) or short form (SF) of HMGA2 growing in semisolid soft agar culture medium. (All images were taken at 40-fold magnification.) Transforming properties of cells transfected with LF, SF or Ev of HMGA2 by CE **(B, D and F)** and soft agar assays **(C, E and G)**. Myometrial primary cells (hMyo) (B and C), myometrial cell line (hUtSMC), (D and E) and myometrial Side Population (MyoSP) cells (F and G).

Battista *et al.*, 1999). The signals and interactions derived by this genetically modified cell population could establish the stem cell fate decisions related to whether a cell self-renews or differentiates into daughter cells (Weissman, 2000; Fuchs *et al.*, 2004).

Our results demonstrate that truncated/short form of HMGA2 (containing only the three DNA-binding domains) has higher transforming activity in hMyo, hUtSMC and MyoSP cells compared with same cells

transfected with full/long form (LF) or empty vector (Ev). Our data support the idea that the formation of the truncated form, disrupts regulatory sequences in the HMGA2 gene resulting in mis-expression of the final transcript (posttranslational modification), as previously described in other benign tumors (Stenman, 2005).

Finally, results obtained from the *in vitro* cell system were translated to *in vivo* animal models. Indeed, transfected hMyo and MyoSP cells were

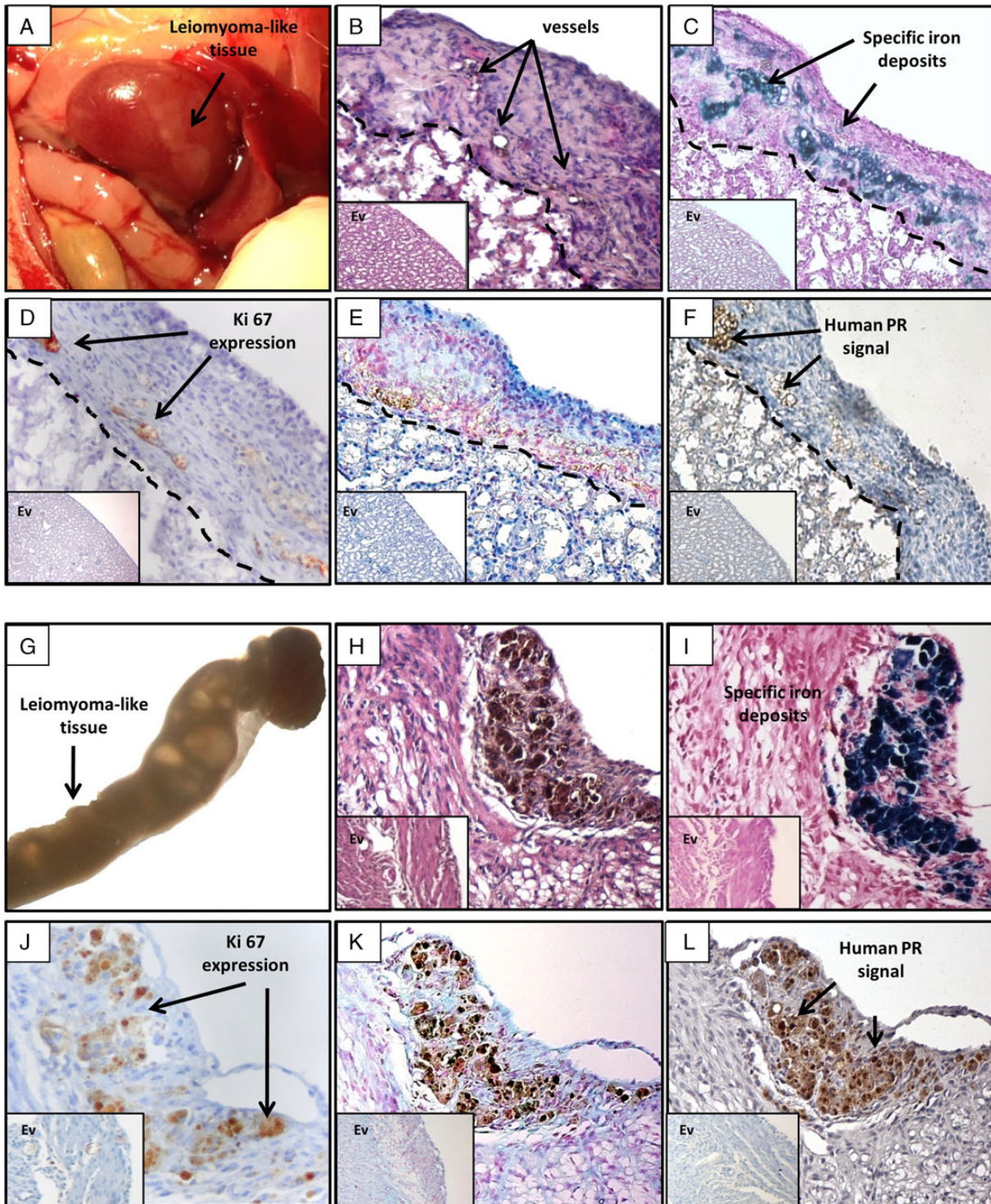


Figure 4 Reconstruction of human leiomyoma-like tissue from truncated/short isoform of High Mobility Group A proteins (HMGA2) in myometrial primary cells (hMyo) and myometrial Side Population (MyoSP) cells in murine kidney capsule and murine uterus, respectively. **(A)** Upper panel macroscopic view shows the xenograft generated in the kidney capsule. **(B)** H&E staining demonstrates the characteristic histology of fibroid-like tissue. **(C)** Prussian blue dye also allowed us to localize the human cells due to the accumulation of iron deposits. Lower panels show **(D)** Ki67 staining, **(E)** Alcian Blue and **(F)** hPR signals in the reconstructed leiomyoma-like tissue, suggesting the regenerative capability from the candidate cells. **(G)** Upper panel macroscopic view shows fibroid-like tissue generated in the uterine horn. **(H)** Histology of fibroid-like tissue was demonstrated by H&E staining. **(I)** Prussian blue dye also allowed us to localize the human cells due to the accumulation of iron deposits. Lower panels confirmed the regenerative capability from the candidate cells in the reconstructed leiomyoma-like tissue by **(J)** Ki67, **(K)** Alcian Blue and **(L)** hPR expression. All images were taking with 10-fold magnification and in all cases Empty vector (Ev) transfected cells were used like negative control.

able to form xenograft leiomyoma-like tissue with high similarity to human fibroids after injection into immunocompromised mice in two different locations. These data provide evidence that indicates an inherent tumorigenic potential, favored also by the administration of steroid hormones (E₂ and P₄), which are essential for the growth and maintenance of the leiomyoma-like tissue generated. The presence of hPR in reconstructed tissues confirms the hypothesis that estradiol could maintain progesterone receptor levels and that it is progesterone through its receptor that promotes leiomyoma growth (Kim *et al.*, 2012). Supporting this proliferative pattern, the Ki67 staining performed in xenograft tissues showed a moderate proliferative model uniquely in the reconstructed areas. Injected human myometrial cells were able to show Alcian Blue staining (typical of ECM components) confirming the nature of the leiomyoma tissue components reconstructed in the animal model.

In summary, these data strongly suggest that truncation of the *HMGA2* gene, resulting in the loss of the two last exons, is sufficient to transform myometrial cells toward leiomyoma cells. Consequently, they were able to develop into fibroids which showed a histopathology that closely resembled that of their human counterpart. Taken together; these findings open up new possibilities to understand the origin of leiomyomas, and subsequently potentially lead to new therapeutic approaches.

Supplementary data

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

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Authors' roles

A.M. and I.C.: study design, execution, analysis, manuscript drafting and critical discussion. A.F.-Á., A.F. and O.B.: execution and analysis. A.D.: study design, execution and analysis. M.C. and C.S.: analysis, manuscript drafting and critical discussion.

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Conflict of interest

None declared.

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