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ARTICLE

microRNA miR-200b affects proliferation, invasiveness and stemness of endometriotic cells by targeting ZEB1, ZEB2 and KLF4

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Abstract Endometriosis is characterized by growth of endometrial tissue at ectopic locations. Down-regulation of microRNA miR-200b is observed in endometriosis and malignant disease, driving tumour cells towards an invasive state by enhancing epithelial-to-mesenchymal transition (EMT). miR-200b up-regulation may inhibit EMT and invasive growth in endometriosis. To study its functional impact on the immortalized endometriotic cell line 12Z, the stromal cell line ST-T1b, and primary endometriotic stroma cells, a transient transfection approach with microRNA precursors was employed. Expression of bioinformatically predicted targets of miR-200b was analysed by qPCR. The cellular phenotype was monitored by Matrigel invasion assays, digital-holographic video microscopy and flow cytometry. qPCR revealed significant down-regulation of *ZEB1* ($P < 0.05$) and *ZEB2* ($P < 0.01$) and an increase in E-cadherin ($P < 0.01$). miR-200b overexpression decreased invasiveness ($P < 0.0001$) and cell motility ($P < 0.05$). In contrast, cell proliferation ($P < 0.0001$) and the stemness-associated side population phenotype ($P < 0.01$) were enhanced following miR-200b transfection. These properties were possibly due to up-regulation of the pluripotency-associated transcription factor *KLF4* ($P < 0.05$) and require attention when considering therapeutic strategies. In conclusion, up-regulation of miR-200b reverts EMT, emerging as a potential therapeutic approach to inhibit endometriotic cell motility and invasiveness.

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Introduction

Endometriosis is a common disease of women at reproductive age that has nowadays reached socioeconomic dimensions. The disease affects 6–10% of women of reproductive age, and has a prevalence as high as 35–50% in women suffering from endometriosis-associated infertility and/or pain (Burney and Giudice, 2012). According to recent studies, endometriosis-related symptoms have a severe impact on both work satisfaction and work ability (De Graaff et al., 2013; Hansen et al., 2013). The indirect costs arising from the number of sick days in addition to direct costs concerning health care services are comparable to the socioeconomic burden caused by other chronic diseases such as diabetes, being estimated as €9579 per year per woman (Simoens et al., 2012). About 50% of affected women often have to undergo recurring surgeries for the removal of active lesions or even hysterectomies (Cheong et al., 2008). Importantly, endometriosis has been linked to female subfertility and infertility (Giudice and Kao, 2004).

The most common hypothesis concerning the pathogenesis of endometriosis was already established in 1927 by John A. Sampson, who elaborated the theory of retrograde menstruation. Endometrial cells would therefore be washed through the Fallopian tubes into the pelvic cavity during menstruation, where they attached themselves to the peritoneal surface (Sampson, 1927). Besides immunological factors (Steele et al., 1984) a possible metaplasia of undifferentiated cells into endometriotic tissue is discussed (Levander and Normann, 1955). Moreover, it has also been suggested that stem cell factors could be involved in the pathogenesis of endometriosis, due to their contribution to unlimited cell proliferation and to a high developmental plasticity (Gargett, 2007; Gargett et al., 2014; Götte et al., 2008, 2011). In the context of a potential stem-cell-dependent pathogenesis of endometriosis, the generation of induced pluripotent stem cells from somatic cells via transduction with the so-called "Yamanaka factors" krupple-like-factor 4 (*KLF4*), octamer-binding-transcription factor 4 (*OCT4*), sex-determining-region-Y (SRY)-box 2 (*SOX2*) and c-Myc has gained considerable attention (Takahashi and Yamanaka, 2006; Yamanaka, 2013). Indeed, it has previously been demonstrated that *SOX2* expression is dysregulated in endometriosis (Götte et al., 2011), and that microRNA (miRNA)-mediated down-regulation of *SOX2*, *KLF4* and *OCT4* in 12Z cells is associated with a reduction of the side population phenotype, a surrogate marker of stemness (Adammek et al., 2013; Greve et al., 2012a). Ectopic tissue is oestrogen-dependent and the tissue shows cyclic hormone-induced changes in animal models (Flores et al., 2007) demonstrating bleeding necrosis, wound healing and fibriosis which is likely to induce symptoms of dysmenorrhoea, chronic pain and dyspareunia. In this context, the concept of epithelial-to-mesenchymal transition (EMT), a well-established mechanism driving tumour progression towards metastasis, has recently gained attention in the context of endometriosis, as it may be linked to migration and local invasion of endometriotic cells at ectopic sites (Bartley et al., 2014; Proestling et al., 2015). In a pathophysiological context, EMT recapitulates developmental processes affecting the cell morphology and polarization as well as migration and invasive capacity that cause a reduced intercellular cohesion and therefore disturb the integrity of an epithelial tissue (Voulgari and Pintzas, 2009). Since cells in endometriotic lesions are capable of invading tissue and therefore of developing deep infiltrating forms of endometriosis, it is a reasonable assumption that endometriosis, although a benign disease, could share some tumour characteristics (Starzinski-Powitz et al., 1999).

In recent years, miRNAs, i.e. small non-coding RNA molecules of approximately 21 nucleotides, have grown in significance for endometriosis research (Neubauer et al., 2012; Ohlsson Teague et al., 2009). Via sequence-specific interactions with target mRNAs, miRNAs can have a profound post-transcriptional impact on gene expression, inducing either RNA degradation, or hampering mRNA translation (Ibrahim et al., 2014). Numerous studies have identified diverging microRNA patterns in ectopic as opposed to eutopic endometrial tissue (Neubauer et al., 2012; Ohlsson Teague et al., 2009). For example, miR-20a is increased in patients with ovarian endometriosis and suppresses netrin-4, with effects on cell cycle progression (Zhao et al., 2014), whereas miR-145 modulates the stem cell phenotype of endometriotic cells by targeting several pluripotency-associated transcription factors, which may promote unlimited proliferation of the lesion at ectopic sites (Adammek et al., 2013). Moreover, a targeting of the proteoglycan Syndecan-1 by miR-10b has been linked to altered invasiveness of endometriotic cells (Schneider et al., 2013), as has the targeting of cytoskeletal elements by miR-145 (Adammek et al., 2013). Most recently, miR-142–3p was identified as a regulator of endometrial stroma cell motility, which was linked to a targeting of the IL-6 signalling pathway (Kästingschäfer et al., 2015).

Another miRNA potentially relevant to invasive growth is miR-200b. miR-200b is down-regulated in the ectopic endometrium compared with the eutopic endometrium of endometriosis patients (Filigheddu et al., 2010; Ohlsson Teague et al., 2009), and in endometrioma compared with healthy endometrium (Hawkins et al., 2011). Indeed, alterations of miR-200b have already been described in the context of the progression of epithelial cancers such as gastric or breast cancer, where they play an important role in the development of invasive carcinomas out of low grade tumours (Korpal et al., 2008; Tryndyak et al., 2010), suggesting that altered miR-200b expression may be linked to the acquisition of a migratory, mesenchymal phenotype. At the molecular level, miR-200b contributes to these changes by targeting the transcription factors ZEB1 and ZEB2, two master regulators of EMT that also control the expression of the antimetastatic adhesion molecule E-cadherin (Christoffersen et al., 2007; Dhayat et al., 2014; Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008). Based on these observations, we postulate that down-regulation of miR-200b may impact on the outcome measure of EMT and therefore on the establishment and cyclical changes in ectopic lesion development in endometriosis. To address this question, the effects of an up-regulated miR-200b expression on endometriotic cell behaviour