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# RESEARCH ARTICLE

# miR-145 suppresses embryo–epithelial juxtacrine communication at implantation by modulating maternal IGF1R

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## ABSTRACT

Successful implantation requires the synchronization of viable embryonic development with endometrial receptivity. The mechanisms allowing for the initiation of crosstalk between the embryo and the endometrium remain elusive; however, recent studies have revealed that there are alterations in endometrial microRNAs (miRs) in women suffering repeated implantation failure and that one of the altered miRs is miR-145. We assessed the role of miR-145 and its target IGF1R, in early implantation. miR-145 overexpression and IGF1R knockdown were achieved in Ishikawa endometrial cells. Quantitative PCR, western blotting and 3′UTR luciferase reporter assays confirmed that IGF1R is a direct target of miR-145 in the endometrium. Attachment of mouse embryos or IGF1-coated beads to endometrial epithelial cells was used to study the effects of altered miR-145 and/or IGF1R expression on early implantation events. miR-145 overexpression or specific reduction of IGF1R impaired attachment in both cases. An IGF1R target protector prevented the miR-145-mediated reduction in IGF1R and reversed the effect of miR-145 overexpression on attachment. The data demonstrate that miR-145 influences embryo attachment by reducing the level of IGF1R in endometrium.

KEY WORDS: IGF1R, Endometrium, Implantation, Embryo, miR-145, miRNA

## INTRODUCTION

Implantation failure is a major cause of infertility in the developed world (Margalioth et al., 2006) and a continuing problem in assisted reproduction (Ferraretti et al., 2012), where success rates after IVF still only lie at around 25% and the rate of embryonic loss at the implantation stage is higher than the rate of later fetal loss (de los Santos et al., 2003; de Mouzon et al., 2010). Although definitions of recurrent implantation failure (RIF) vary (Coughlan et al., 2014; Polanski et al., 2014), it is clear that embryo transfer commonly leads to a biochemical pregnancy [human chorionic gonadotropin (hCG) detected but no embryonic sac] or no detectable pregnancy at all. RIF appears distinct from recurrent miscarriage; that is, there is a group of women in whom pregnancy repeatedly fails very early on (Koot et al., 2011). This might be accounted for by the repeated transfer of karyotypically abnormal embryos or, alternatively, by a lack of adequate receptivity in the endometrium.

Prior to the arrival of an embryo, the action of steroid hormones on endometrium produces a period of receptivity, referred to as the 'window of implantation' (Cha et al., 2012; Psychoyos, 1974; Tabibzadeh et al., 1987). In humans, this lasts from approximately day 20 to day 24 of the menstrual cycle (Achache and Revel, 2006; Aplin, 2000; Aplin and Kimber, 2004; Bergh and Navot, 1992; Enders, 1989; Psychoyos, 1974). After the endometrium becomes receptive and the embryo reaches the blastocyst stage, the embryo must initiate interaction with the luminal epithelium (Dey, 2004; Nimbkar-Joshi et al., 2009; Rashid et al., 2011; Tabibzadeh et al., 1987). A cascade of physiological and molecular events is then triggered, leading to the establishment of a stable maternal–conceptus relationship (Garrido-Gomez et al., 2010; Simón et al., 2000a; Simón et al., 2000b). It is estimated that half of implantation failures in women suffering from RIF after embryo transfer are due to defects in endometrial receptivity. However, little is known about the molecular events that establish receptivity prior to implantation, especially in humans (Aplin, 2007), or the mechanisms that mediate early dialogue between the embryo and endometrium (Bazer et al., 2009; Cakmak and Taylor, 2011; Salker et al., 2011).

Analysis of temporal changes in biopsies of endometrial tissue taken from different stages of the menstrual cycle and from patients with RIF has been suggested as a means by which to identify candidate molecules that are involved in mediating endometrial receptivity. 'Omics' technologies have allowed the identification of candidate mediators of adhesion (Singh and Aplin, 2014), menstrual-cycle-related alterations and illuminated adverse effects of some ovarian stimulation regimes (Altmäe et al., 2014; Aplin and Singh, 2008; Domínguez et al., 2009; Rai et al., 2010; Vilella et al., 2013), but these have not yet been exploited in new medical treatments to improve endometrial receptivity.

MicroRNAs (miRs) suppress gene expression at the posttranscriptional level by blocking target mRNA translation or selecting mRNA for degradation (Bartel, 2004). It has been suggested that they are involved in mediating endometrial

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responses to maternal hormones and regulating the transition from the proliferative into the secretory phase (Kuokkanen et al., 2010). Endometrial miRNA profiles are altered in women suffering from endometriosis – a disease associated with infertility (Burney et al., 2009; Burney et al., 2007) – and in patients with RIF (Revel et al., 2011), suggesting that they might be important regulators of endometrial receptivity and thus implantation success.

Among the most dysregulated miRs within the endometrium of women with RIF is miR-145 (Revel et al., 2011). We have recently shown that miR-145 is important in mediating placental growth in humans (Farrokhnia et al., 2014; Forbes et al., 2012). One of the targets of miR-145 is the type-1 insulin-like growth factor receptor (IGF1R) (La Rocca et al., 2009; Law et al., 2012) and IGF1R mRNA has been previously shown to be present in endometrium (Zhou et al., 1994). Despite this, to our knowledge, its role in regulating human endometrial receptivity or implantation remains to be explored.

We hypothesized that altered expression of miR-145 contributes to the initial maternal–embryo interaction during the window of implantation by regulating gene expression in endometrial epithelial cells. To explore this, we manipulated miR-145 expression in endometrial cells and assessed embryo attachment using *in vitro* models (Kang et al., 2014). We examined IGF1R as a potential target of miR-145 in epithelium and the effect of altering the level of the receptor on embryo attachment. We suggest that IGF1R plays a role in implantation.

## RESULTS
### miR-145 overexpression prevents embryo attachment
Following transfection with miR-145-specific miR mimics, levels of miR-145 were significantly increased compared to control (10–100 nM; $P<0.05$, $n=6$) (Fig. 1A). The transfection procedure alone or the presence of a non-targeting miR mimic had no effect on levels of miR-145. All subsequent experiments were undertaken using both 50 nM and 100 nM of the mimetic. A total of 18 5-day-old mouse embryos were transferred to Ishikawa cells 48 h after transfection with miR-145. The stability of embryo attachment was measured after a further 24 h of co-culture (Carver et al., 2003). Displacement or disruption of cells surrounding the attached mouse embryos, and marked outgrowth of trophoblast cells were observed in the controls, whereas embryos attached to miR-145-transfected cells retained the blastocyst morphology including a clearly defined cavity (Fig. 1B). Embryo attachment stability was measured using a five point scale: 1, floating; 2, weakly attached but detached after tapping; 3, weakly attached but stuck at the attachment site after tapping; 4, stably attached; and 5, stably attached and showed outgrowth (Kang et al., 2014). This quantification revealed that embryos on control cells had attachment scores of 3–5 compared to scores of 1–2 for miR-145-treated cells. Control cells without any transfection, and mock or pre-miR transfected cells were significantly ($P=0.0097$) more stably attached than on miR-145 transfected cells (Fig. 1C).

### miR-145 regulates IGF1R expression in Ishikawa cells
miR-145 has multiple targets, one of which is IGF1R, which is present in the human endometrium (Zhou et al., 1994). Ishikawa cells were transfected with a miR-145 mimic and the effect on IGF1R mRNA and protein monitored using quantitative real-time PCR (QPCR) and western blotting, respectively. Despite no changes in IGF1R mRNA (Fig. 2A),
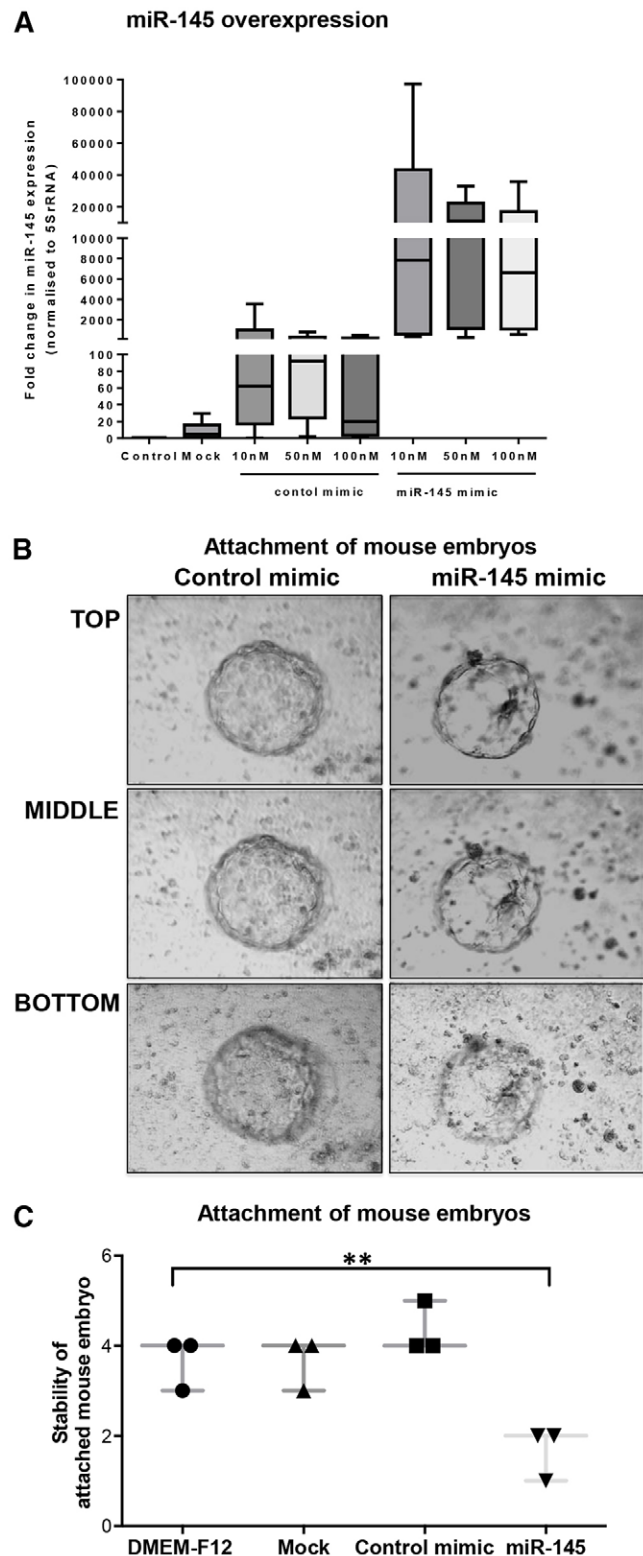


Fig. 1. See next page for legend.

IGF1R protein expression was reduced 48 h following miR-145 overexpression (Fig. 2B).

To determine whether miR-145 directly binds to IGF1R in these cells, the highly conserved predicted binding site within the IGF1R 3′UTR (3804–3810 bp) was cloned into the

**Fig. 1. miR-145 regulates embryo attachment.** Ishikawa cells were mock-transfected (mock) or transfected with a non-targeting control miR-mimic (control mimic) or a miR-145 mimic (10–100 nM). (A) Overexpression of miR-145 was confirmed by QPCR. ***$P<0.0001$ ($n=6$, Wilcoxon Signed Rank test in comparison to control). The box represents the 25–75th percentiles, and the median is indicated. The whiskers show the range. (B) Phase-contrast images at three z-planes of mouse embryos attached to Ishikawa endometrial cells following transfection with miR-mimics. Images were taken after 24 h of co-culture (representative of $n=15$). (C) The stability of mouse embryo attachment to Ishikawa cells was assessed as previously described (Kang et al., 2014). Data are expressed as median and interquartile range. **$P<0.01$ ($n=3$, Kruskal–Wallis test with Dunn's multiple comparison).

pmiR-GLO luciferase reported vector alone or in combination with miR-145 mimics (Fig. 2C). As expected, luciferase activity was detected under all conditions in the presence of a non-targeting mimic. In the presence of the miR-145 mimic there was an 81% reduction in luciferase activity of the IGF1R 3804–3810 vector (Fig. 2C; $P=0.0079$, $n=5$). Co-transfection of the miR-145 mimic with the empty pmiR-GLO (empty vector) or the scrambled IGF1R 3′UTR vector (IGF1R Δ3804–3810) did not alter luciferase activity. This indicates that miR-145 binds directly to the 3804–3810 region in IGF1R 3′UTR.
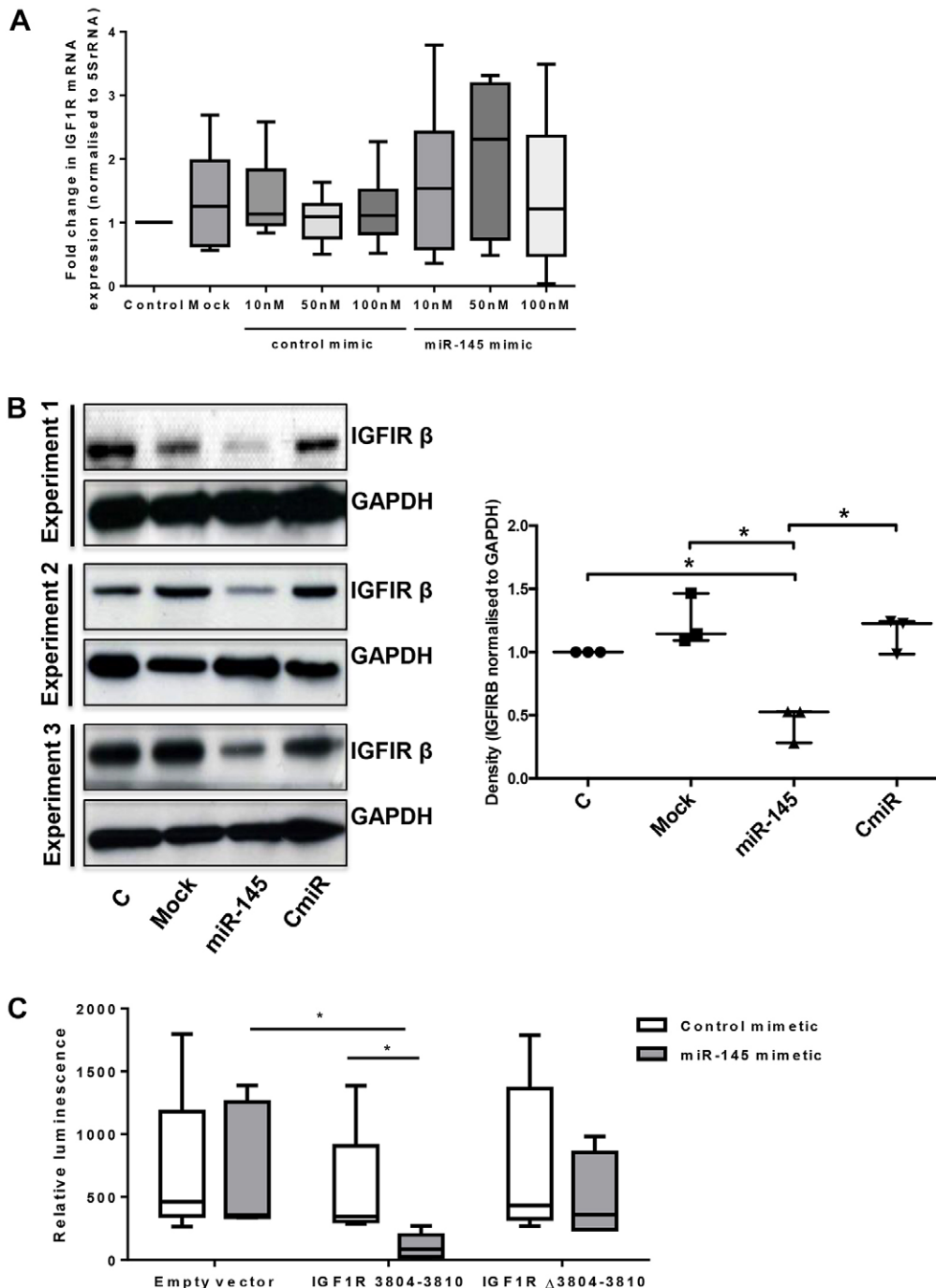


**Fig. 2. miR-145 regulates IGF1R expression.** (A,B) Ishikawa cells were transfected with a non-targeting (control mimic) or miR-145 specific miR mimic (10–100 nM) and appropriate controls. (A) IGF1R mRNA expression was quantified by QPCR and normalized to β-actin mRNA. Data are presented as a fold change in comparison to control ($n=6$). There is no observed change in mRNA expression. (B) Western blotting revealed decreased expression of IGF1R protein following transfection with miR-145 mimic at 100 nM in comparison to control. Blots were stripped and re-probed with anti-GAPDH antibodies to correct for protein loading. Blots represent at least three individual sample sets. Data are expressed as median and interquartile range. (C) Synthetic oligonucleotides for the specific (IGF1R 3804–3810) or mutated (IGF1R Δ3804–3810) miR-145-binding sites in IGF1R mRNA, were cloned in to pmiR-GLO luciferase reporter vector and transfected in to Ishikawa cells in the presence of non-targeting (control mimic) or miR-145 specific miR-mimics. Direct interaction between miR-145 and IGF1R in Ishikawa cells was assessed by luciferase reporter assay. Levels of firefly luciferase were assessed and normalized to levels of β-galactosidase. *$P<0.05$ ($n=5$, Kruskal–Wallis test). For A and C, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the range.

## IGF1R is upregulated in the endometrial epithelium at the time of endometrial receptivity

Previous studies have localized IGF1R mRNA *in vivo* to endometrial luminal and glandular epithelium (Zhou et al., 1994) but localization of IGF1R protein has not been reported. We examined IGF1R protein expression in human endometrium at different time points in the menstrual cycle (proliferative, days 7–17; early secretory, days 18–20; mid-secretory, days 20–21; late secretory, days 26–27). IGF1R was detectable at all stages (Fig. 3A,C,E,G). Some regional variation in epithelial staining was evident, but staining intensity was highest at the endometrial luminal epithelium during the mid and late secretory stages (Fig. 3E,G).

## miR-145 overexpression prevents IGF1-loaded bead attachment

Multiple factors are released from embryos and/or are present in the uterine fluid, including the predominant ligand for IGF1R, IGF1 (Kane et al., 1997). To demonstrate functional receptor at the apical epithelial surface, and to assess the possibility that IGF1R might contribute directly to an adhesive interaction at the cell surface, we examined attachment to confluent Ishikawa cells of IGF1-coated embryo-sized beads (Kang et al., 2014). Attachment of beads was assessed by counting the number stably attached against the total number of beads originally transferred (Kang et al., 2014). Following overexpression of miR-145 (48 h), IGF1 or BSA (negative control)-loaded beads were transferred and, 24 h later, attachment was assessed. 52% of beads carrying IGF1 were stably attached under control conditions (Fig. 4) and following miR-145 overexpression this was reduced to 17% (100 nM; $P<0.01$; $n=5$). Levels of attachment were unaffected by the transfection procedure (mock) or in the presence of non-targeting miR (control mimic). Levels of attachment of BSA-coated beads were low and unaffected by miR-145 overexpression.
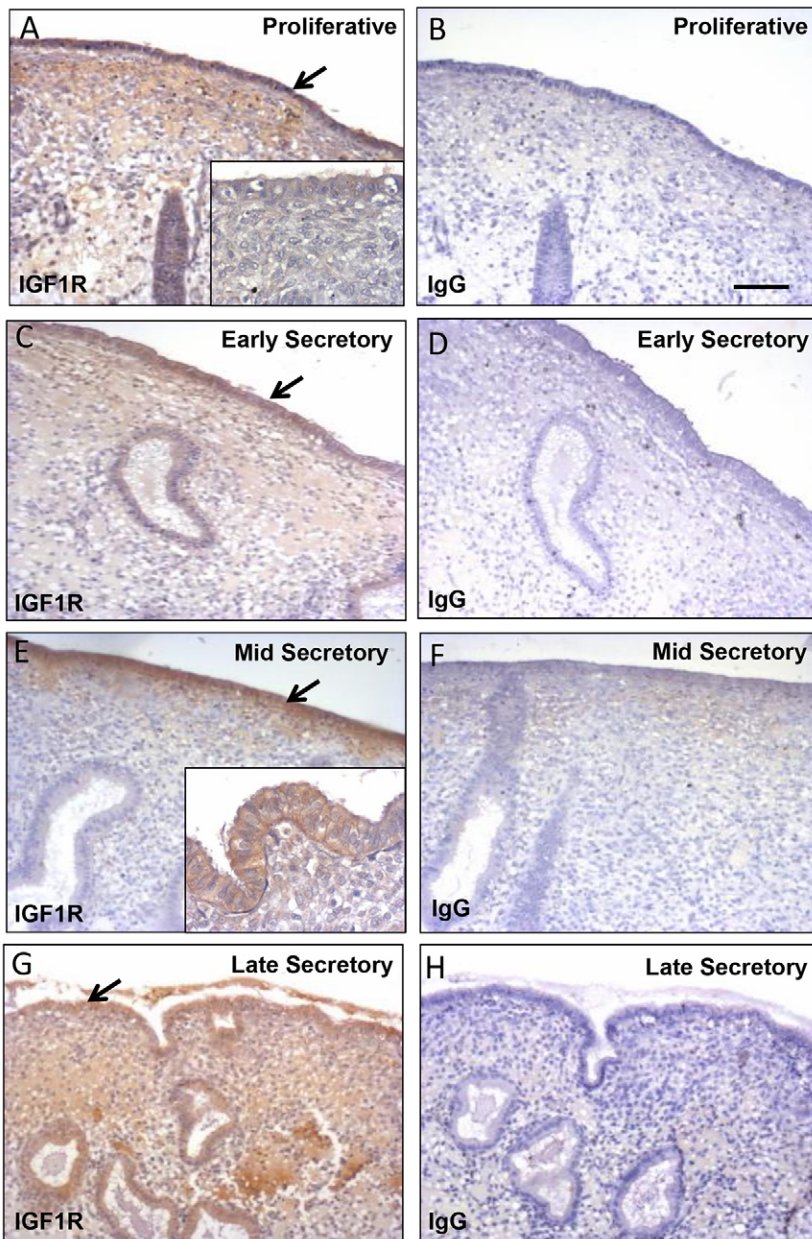


**Fig. 3. IGF1R is present in endometrial luminal epithelium at the receptive phase.** Endometrial biopsies obtained from different stages of the menstrual cycle were stained for IGF1R. Mouse IgG was included as a negative control in adjacent sections. IGF1R is expressed in the endometrium throughout the menstrual cycle but appears to be primarily localized to the endometrial epithelium (arrows) in the mid-late secretory stages, the former representing the time of optimal endometrial receptivity. Insets in A and E show luminal epithelium at higher magnification. Each image is representative of at least three tissue samples. Scale bar: 75 μm. Insets are 75 μm across.
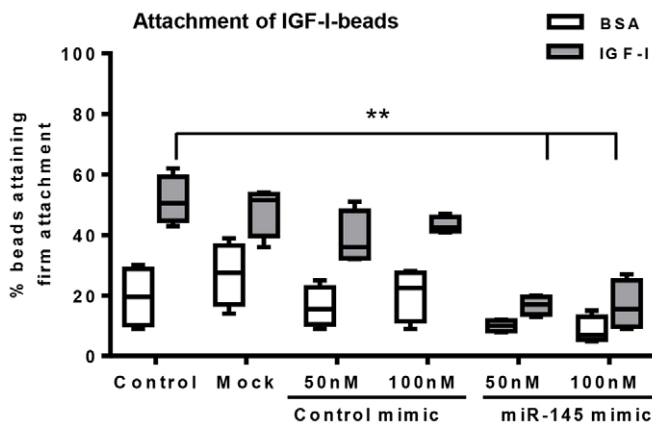
**Fig. 4. miR-145 overexpression reduces IGF1-coated bead attachment.**
Ishikawa cells were mock-transfected, transfected with a non-targeting
control miR-mimic or a miR-145 mimic (10–100 nM) and cultured for 48 h
prior to transfer of beads coated in IGF1 (IGF-I) or BSA (negative control).
Attachment was assessed after a further 48 h using a scale adapted
from Kang et al., 2014. Data are expressed as a percentage of total
beads. The box represents the 25–75th percentiles, and the median is
indicated. The whiskers show the range. **$P < 0.01$ ($n = 5$, Kruskal–Wallis
test).

## siRNA-mediated knockdown of IGF1R suppresses embryo attachment

The role of IGF1R in mediating embryo attachment was
examined using small interfering RNA (siRNA) (100 nM) to
directly reduce its expression in endometrial cells. Western
blotting confirmed IGF1R expression to be reduced by 80% after
48 h (Fig. 5A,B). Attachment of mouse embryos (Fig. 5C,D) or
IGF1-coated beads (Fig. 5E) was reduced following IGF1R
knockdown ($P < 0.05$). To determine whether the effect of miR-
145 on attachment is mediated by its ability to target IGF1R, we
prevented the interaction of miR-145 with the IGF1R 3′UTR
using the specific miR-target protector for the 3804–3810 region
of IGF1R mRNA (Fig. 5F,G). miR-145 mimics (50 nM)
significantly reduced IGF1R protein (Fig. 5F) and attachment
of IGF1-coated beads (Fig. 5G) compared to non-targeting miR
mimics (control mimic). Western blotting (Fig. 5F) demonstrated
that miR-145 was unable to reduce levels of IGF1R in the
presence of the target site-specific target protector (IGF1R TP
3804–3810). The ability of miR-145 to reduce attachment of IGF-
I-loaded beads was significantly attenuated in the presence of the
target-site-specific target protector (IGF1R TP 3804–3810;
100 nM; $P < 0.05$) but was unaffected by the scrambled IGF1R
target protector (Δ3804–3810; 100 nM) (Fig. 5G). This suggests
that the effects of miR-145 on embryo attachment are directly
mediated by IGF1R.

## DISCUSSION

Array approaches are cataloguing biomarkers of receptivity,
comparing endometrium from different stages of the menstrual
cycle or from fertile and infertile women (Altmäe et al., 2010;
Ruan et al., 2013), but data are difficult to interpret in the absence
of functional information on the role of putative markers in
the process of implantation. Comparison of miR profiles of
endometrium from RIF and normally fertile women yielded a
group of candidates among which miR-145 showed an
~threefold elevation (Revel et al., 2011), leading to a
hypothesis that among its targets are components vital for
implantation. However, the cell populations expressing miR-145

were not identified. By using an embryo attachment model, we
now demonstrate that overexpresssion of miR-145 at levels
approximately comparable to those reported in RIF endometrium
reduces the stability of the embryo–epithelial interaction. Given
the barriers to direct examination of human embryo implantation,
*in vitro* modelling is a viable approach to gathering relevant
functional information to complement the analytical data
emerging from arrays. Translated to the *in vivo* situation, we
predict that such an effect might delay implantation to the point
where corpus luteum rescue would not occur, leading to a failed
pregnancy. Data from the rat similarly point to a role for miRs in
regulating endometrial cell function in the implantation window
(Xia et al., 2014a; Xia et al., 2014b). Such a prediction in human
must be tempered by the limitations of the *in vitro* model, which
includes the use of mouse embryos and cells from a well-
differentiated adenocarcinoma. However, a recent proteomics
study of apically displayed glycoproteins in polarized Ishikawa
cells showed that these were substantially similar to the
epithelium *in vivo* (Singh and Aplin, 2014). Furthermore we
have observed that hatched human blastocysts attach to these
cells with kinetics that are not dissimilar to those of the mouse
(unpublished).

Following previous reports that IGF1R is a target gene for
miR-145 (La Rocca et al., 2009; Law et al., 2012), we have
shown that overexpression of miR-145 reduces the level of
IGF1R protein in endometrial epithelial cells, and identified a
target site in the 3′UTR. This is consistent with miR action in
mammalian cells through translational repression (Erson and
Petty, 2008). A previous study reported mRNA encoding IGF1R
in the endometrial luminal epithelium during the mid-late
secretory phase (Zhou et al., 1994). We have here extended
these data to show the distribution of the protein through the
normal cycle and, most importantly, its expression in mid
secretory luminal epithelium. Direct evidence for hormonal
control of IGF1R expression comes from studies in isolated
human endometrial cells that report IGF1R downregulation by
progesterone (Strowitzki et al., 1996). In rats, IGF1R expression
in the uterus is enhanced by estrogen treatment (Ghahary and
Murphy, 1989) and in baboons, expression at the glandular and
luminal epithelium undergoes cyclic changes and is upregulated
by estrogen (Hild-Petito et al., 1994).

Our data reveal that there might be a previously unsuspected
role for IGF1R in regulating embryo attachment, with stability of
adhesion to the epithelium dropping after receptor knockdown in
a fashion that parallels the effect of miR-145 overexpression.
IGF1R is a protein tyrosine kinase that is predominantly activated
by IGF1 and IGF2. Immunoblotting identified IGF1R as being
expressed in Ishikawa cells, so we adopted a ligand-coated bead
assay (Kang et al., 2014) to probe its presence and function at the
apical epithelial surface where embryo attachment is initiated.
The observations that IGF1-coated beads can attach stably and
that IGF1R knockdown impairs this process, might indicate the
presence of receptor at the apical cell surface and, perhaps
surprisingly, its ability to mediate an adhesive interaction with an
object that approximates the size of the implanting blastocyst. By
specifically inhibiting the interaction between miR-145 and
IGF1R 3′UTR, without affecting interaction between miR-145
and other targets, we were further able to attribute the effects of
miR-145 on embryo attachment to interaction with IGF1R.
Previous work in receptive-phase mouse uterus has shown that
IGF1 delivered on the surface of a bead could activate a response
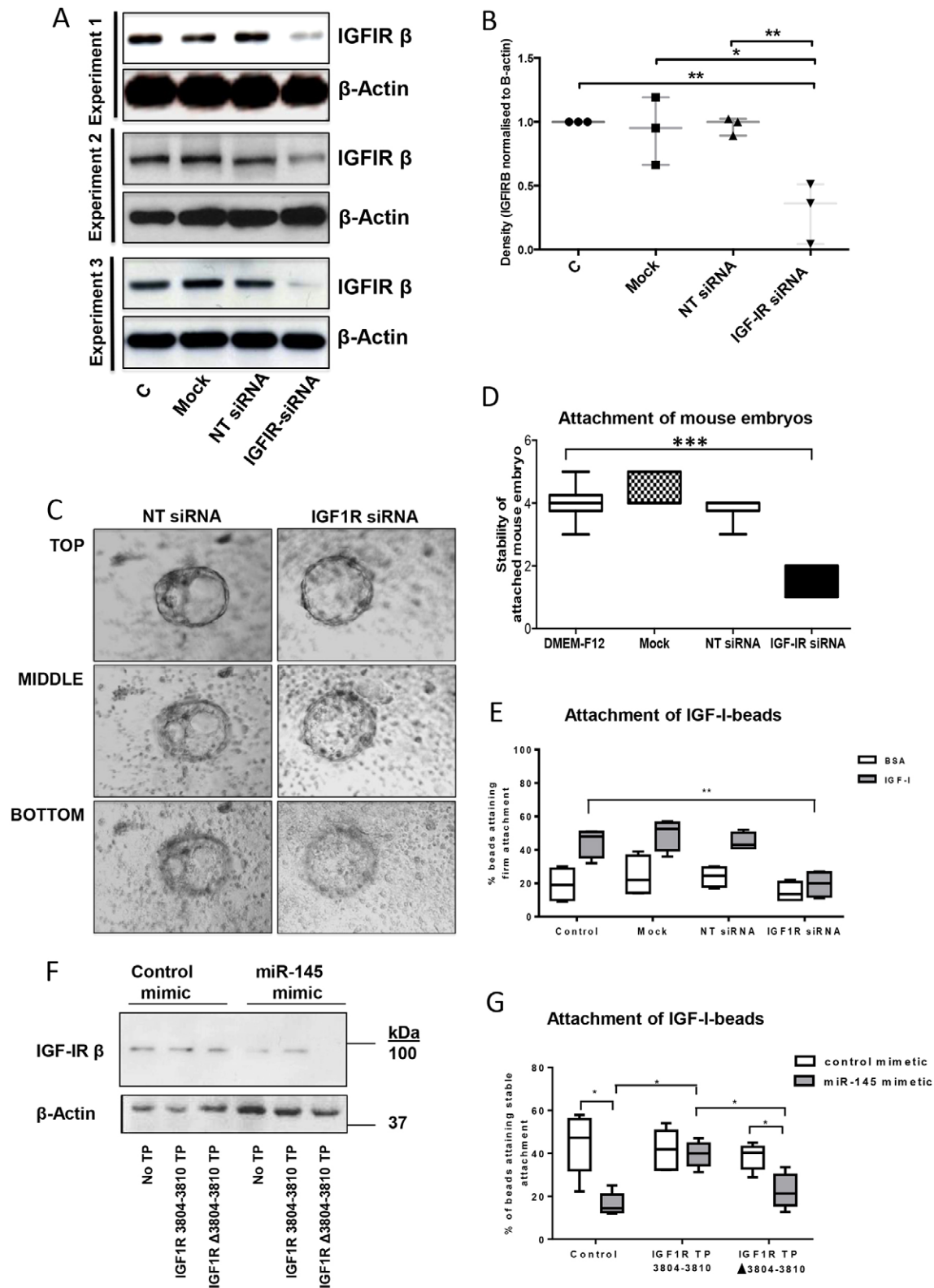in the epithelium that was transduced to stroma to influence

**Fig. 5.** See next page for legend.

vascular permeability (Paria et al., 2001). Our data further validate the ligand-coated bead interaction model as a tool to investigate expression and function of apical epithelial receptors.

Although IGF2 is expressed by both the mouse and human blastocyst (Lighten et al., 1997; Szabó and Mann, 1995), there is no evidence at present that surface-associated IGFs are displayed on trophectoderm. IGF ligands, however, are produced

**Fig. 5. The effects of miR-145 on embryo attachment are mediated by IGF1R.** Non-targeting (NT) or IGF1R-specific siRNA (100 nM) were transfected into Ishikawa cells. Untreated ('C') and mock-transfected cells were included as controls. (A) Knockdown was confirmed by western blotting. Membranes were re-probed for β-actin to control for protein loading. (B) Density of bands was quantified, and levels were normalized to β-actin and expressed as a fold change relative to control. *$P<0.05$, **$P<0.01$ ($n=3$, one-way ANOVA with Tukey's multiple comparison test). Data are expressed as median and interquartile range. (C–E) Cells transfected with IGF1R siRNA or appropriate controls (NT siRNA) were used for *in vitro* implantation using (C–D) mouse embryos or (E) ligand-coated beads [IGF1 (IGF-I) or BSA]. (C) Phase-contrast images of mouse embryos attached to Ishikawa cells after knockdown of IGF1R at three z-plane levels, taken 24 h after transfer of embryos ($n=15$). (D) The stability of mouse embryo attachment to Ishikawa cells following transfection with IGF1R siRNA. ***$P<0.001$ ($n=6$, Kruskal–Wallis with Dunn's multiple comparison). (E) The effect of IGF1R knockdown on firm attachment of IGF1-coated beads was monitored. IGF1R knockdown resulted in a reduction in IGF1-coated bead attachment in comparison to controls **$P<0.01$ ($n=5$, Kruskal–Wallis test). (F–G) Ishikawa cells were transfected with a specific miR target protector (TP) (100 nM) for the miR-145-binding site at the 3804–3810 bp region of IGF1R 3′UTR (IGF1R TP 3804–3810) or with a scrambled sequence for this region (IGF1R TP Δ3804–3810), which served as a negative control in the presence of a non-targeting control mimic (control mimic) or miR-145 specific miR mimic (miR-145 mimic). (F) Western blotting confirmed that the IGF1R 3′UTR IGF1R target protector (3804–3810) prevented miR-145 induced reduction in IGF1R protein expression after 48 h. (G) Analysis of stable attachment of IGF1-coated beads was assessed after a subsequent 48 h. BSA-coated beads were included as a control. * $P<0.05$ ($n=5$, Kruskal–Wallis test). For D,E,G, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the range.

endogenously in the endometrium (Giudice et al., 1993; Murphy and Ghahary, 1990). One hypothesis consistent with these data is that IGF2 released by the implanting embryo activates IGF1R locally in epithelial cells, and signals ensue through the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways to influence downstream factors regulating attachment (Forbes and Westwood, 2008; Forbes et al., 2008). For example, in breast cancer cells IGF1R activation upregulates fascin-1, a modulator of adhesion and cell–cell interactions (Wong et al., 1999) to promote reorganization of cell–cell contacts (Guvakova et al., 2002a; Guvakova et al., 2002b; Yamashiro et al., 1998). Increased expression of fascin has been reported at implantation sites in mice, suggesting that it has a potential role in the initial adhesion stages (Yoon et al., 2004).

However, in recent years it has emerged that binding and crosstalk between IGF1R and cell–cell or cell–matrix adhesion molecules can occur in either the presence or absence of IGF ligand, including with integrin αvβ3 (Maile et al., 2008; Xi et al., 2008), which is involved in implantation (Kang et al., 2014; Lessey, 1997). Such interactions are important for cytoskeletal organization, cell adhesion and motility (André et al., 1999; Clemmons and Maile, 2005; Juliano and Haskill, 1993; Mauro and Surmacz, 2004; Tai et al., 2003). Further work is therefore required to examine the possibility that cis interactions between growth factor receptors and adhesion molecules might occur at the apical luminal epithelial plasma membrane during implantation.

miRs alter the expression of target genes in one of two ways depending on the level of base complementarity with the 3′UTR of the target mRNA. Complete complementarity results in degradation of target, whereas partial complementarity induces translational repression (Filipowicz et al., 2008). Partial complementarity enables miRs to regulate expression of

multiple targets (Erson and Petty, 2008), and despite our miR-145 target blocking data demonstrating that IGF1R mediates the effects of miR-145 on embryo attachment, we cannot rule out that miR-145 has effects on implantation by targeting genes other than IGF1R. For example, miR-145 targets MUC-1 in the human embryonic kidney cell line HEK293 (Sachdeva and Mo, 2010); MUC-1 is expressed on the luminal epithelium of the endometrium (Hey et al., 1994) and is cleared at the site of implantation as a result of local juxtacrine signaling of the embryo (Singh et al., 2010). In both Ishikawa cells and primary endometrial cells, miR-145 regulates the expression of fascin (Adammek et al., 2013; Götte et al., 2010).

miR-145 is a regulator of endometrial IGF1R expression, and our *in vitro* model has suggested that IGF1R has a role in implantation. IGF1R is upregulated at the time of embryo attachment *in vivo* and reduced expression in epithelial cells destabilizes embryo attachment *in vitro*. Reduced IGF1R expression has been reported in mid-secretory endometrium in women with unexplained infertility (Wu and Zhou, 2004). There is evidence for regulation by leukemia inhibitory factor (LIF), which is required for endometrial receptivity in mice (Rosario et al., 2014). miR-145 in endometrium might be sensitive to steroid hormone – whereas estrogen influences expression in mouse splenic lymphocytes (Dai et al., 2008), it is under the control of progesterone in murine endometrial epithelial cells (Yuan et al., 2014). We have yet to establish whether miR-145 is under hormonal control in the human endometrium, but future studies will explore the possibility of utilizing synthetic steroids to modulate expression. We note the therapeutic potential of targeted miR-145 inhibitors (Caruso et al., 2012) and suggest that therapies to suppress miR-145 in the endometrium might improve pregnancy rates in women with RIF.

## MATERIALS AND METHODS
### Endometrial tissue
Endometrial biopsies were from pre-menopausal women at different points in the menstrual cycle were obtained anonymously from local pathology archives. Research access to the pathological archive of St Mary's Hospital was approved by Central Manchester REC. No signs of hyperplasia, neoplasia or inflammation were present in these samples. Dating of the menstrual cycle was established from histopathology reports and was confirmed by histological examination of sections stained with haematoxylin and eosin.

### Cell culture
Ishikawa cells (a well-differentiated human endometrial adenocarcinoma line), obtained from American Type Cell Culture (ATCC), were maintained in phenol red-free DMEM/F12 medium (Invitrogen, UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were grown in 95% air and 5% $CO_2$ on matrigel (1:8 dilution; growth factor-reduced, BD, UK) for further attachment assays.

### Animals, superovulation and embryo recovery
All experiments were conducted under a Home Office license and the Animal Act, 1986, and had local ethical approval for care and use of laboratory animals. C57BL/6 strain mice were maintained by the Biomedical Services Unit at the University of Manchester. Mice were kept under standard environmental conditions of 12 h light and 12 h dark and housed at 20–22°C and 40–60% humidity with food and water provided at ad libitum and males (less than 6 months old) were caged singly. Female mice (6–8 weeks) were superovulated with 5 IU of pregnant mare serum gonadotrophin (PMSG, Calbiochem, Nottingham, UK) and ovulation was synchronized by 5 IU human chorionic gonadotrophin (hCG, Intervert UK, Milton Keynes, UK) 46–48 h later, both by intraperitoneal injection. Females were placed singly with males

of the same strain overnight. The presence of a vaginal plug the following morning (day 1 of pregnancy) was used as an indicator of successful mating. Pregnant mice were killed on day 2, 48 h after hCG injection. Using sterilized scissors and forceps the lower abdominal cavity was incised and both oviducts dissected. Two-cell embryos were obtained from the oviduct either by flushing using a 34G blunt-ended stainless steel needle (Cooper Needleworks, Birmingham, UK) and syringe. Flushed embryos were washed with M2 media (Sigma) supplemented with 4 mg/ml BSA washed in KSOM (Millipore, UK) and cultured in a 30 µl drop of KSOM covered with mineral oil at 5% $CO_2$, 37℃ until the blastocyst stage. Only expanded blastocysts with clearly observable inner cell mass and trophectoderm on day 5 were included in the study.

### IGF1R localization
Sections of endometrial tissue (5 µm thick), or Ishikawa cells fixed in 4% PFA were incubated overnight at 4℃ in a humidity chamber, with either rabbit polyclonal anti-IGF1R antibody (1:75; Cell Signaling Technologies, 3027) or IgG purified from rabbit serum (1:2000; Cell Signaling Technologies) followed by anti-rabbit biotinylated antibody (1:200) (Dako), avidin peroxidase and then developed by DAB as previously described (Forbes et al., 2008).

### Overexpression of hsa-miR-145 in Ishikawa cells
Non-targeting (Cy3-labeled Pre-miR precursor negative control) or miR-145 specific miR mimics (Pre-miR mimics; Ambion, UK) were transfected into Ishikawa cells (10–100 nM) with DharmaFECT2 reagent (Dharmacon, UK) as previously described (Kang et al., 2014). Following transfection, cells were cultured for up to 96 h in 20% $O_2$ at 37℃. Prior to analysis in subsequent experiments, overexpression was confirmed by QPCR. The effect of miR-145 overexpression was compared with that of three controls: untreated cells, tissue exposed to transfection procedure alone (mock) or tissue transfected with non-targeting miR mimic (Pre-miR precursor negative control, Ambion, UK) which has no known homology to any mammalian miRs.

### siRNA mediated knockdown of IGF1R
siRNA sequences used to target the human IGF1R gene (GenBank NM-000875) were either in a SMARTPool containing four different target sequences: A, 5′-GGAAGCACCCUUUAAGAAU-3′; B, 5′-GGACUC-AGUACGCCGUUUA-3′; C, 5′-AAAUACGGAUCACAAGUUG-3′ and D, 5′-AGUGAGAUCUUGUACAUUC-3′ (100–500 nM; Dharmacon, UK), or using a single validated sequence 5′-GGAAUACAGGAA-GUAUGGA-3′ (100–500 nM; Ambion, UK). Silencer Select Negative Control siRNA (Ambion, UK), which does not target any known sequence in the human genome, was used in control experiments. The specific and non-targeting siRNA sequences were transfected into Ishikawa cells using DharmaFECT 2 reagent as previously described (Kang et al., 2014).

### Quantitative RT-PCR based analysis of miR expression
Total RNA was extracted from Ishikawa cells from at least six separate experiments by using the miRVANA total RNA extraction kit (Ambion, UK) and quantified using a Quant-iT Ribogreen kit (Molecular Probes). The expression of miRs was assessed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) following the manufacturer's instructions. Briefly, 25 ng RNA was reversed transcribed using Universal cDNA Synthesis kit (Exiqon), which allows polyadenylation and reverse transcription of all miRs within the sample into cDNA in a single reaction step. Individual miRs were then detected using LNA-enhanced miRNA-specific primer sets (Exiqon) for hsa-miR-145 (target sequence: 5′-GUCCAGUUUUCCCAGGAAUC-CCU-3′) or 5S rRNA. miR expression was quantified against standard curves generated from human reference total RNA (Stratagene, USA). PCRs were performed in duplicate for each sample to control for user error, using a Stratagene MX3000P real-time PCR machine. Values were only deemed acceptable when each replicate sample deviated by less than 1 from its matched duplicate. The CT value for each sample (i.e. the average of the two duplicates) was used to calculate absolute values (determined from the standard curve). To further control for variations in

sample input into the PCR assay, levels of miR-145 were normalized to 5S rRNA, which is present at equal levels in all our samples (data not shown). Data were then expressed as fold change compared to control and analysed using the Wilcoxon signed-rank test.

### Quantitative RT-PCR based analysis of mRNA expression
Total RNA was extracted from Ishikawa cells by using the miRVANA total RNA extraction kit (Ambion UK) and quantified using a Quant-iT Ribogreen kit (Molecular Probes). 100 ng of total RNA from each sample was reverse transcribed using AffinityScript cDNA synthesis kit (Stratagene, USA) and IGF1R and β-actin mRNA was amplified by QPCR using 200 nM primers 55℃ [IGF1R: forward, 5′-GAAGGAGGAGGCTGAATAC-3′; reverse, 5′- CTACAACATCAC-CGACC-3′; Invitrogen (Paisley, UK); β-actin: forward, 5′-AGCCACCCCACTTCTCTCTAA-3′, reverse, 5′-ACACGAAAGCA-ATGCTATCACCT-3′ (MWG Biotech, UK)] and Stratagene Brilliant SYBR Green II QPCR mastermix, with 5-carboxy-x-rhodamine as a passive reference dye in a Stratagene's MX3000P real time PCR machine. mRNA expression was quantified against standard curves generated from human reference total RNA (Stratagene, USA). Data were normalized to β-actin mRNA then expressed as a percentage of levels measured in cells transfected with pre-miR negative control mimic, and analysed using the Wilcoxon signed-rank test.

### In vitro implantation models
#### Embryo attachment assay
Mouse embryos (5 days old) were transferred onto Ishikawa cells in independent microwells of a 96-well plate. Multiple observations of attaching embryos from 24–48 h after transfer allowed distinct stages of attachment to be identified. A standardized plate movement protocol was implemented to characterize the stability of attachment: the plate was moved quickly three times laterally and then orthogonally to detect unattached embryos. Upon moving the plate, unattached embryos floated, or rolled, over the epithelial surface. Attached embryos were then examined for tandem movement when the microscope stage was tapped. At initial attachment, embryo wobble was observed, whereas at more advanced stages of attachment, embryos moved fully in concert with the underlying epithelial layer. Five stages of attachment were defined and used as a measurement scale as previously described (Carver et al., 2003; Kang et al., 2014).

#### Bead attachment assay
10 µl of embryo-sized beads (Bio-Rad) were washed six times in sterile PBS. Beads were then incubated with 20 µl of 100 ng/µl IGF1 (R&D) and BSA (RIA Grade, Cat no: A7888, Sigma) dissolved in PBS, and overlaid with 1 ml of mineral oil (Sigma) at 37℃ for 2 h. Beads were used immediately after incubation. Prior to co-culture, the mineral oil was removed and the beads were washed in sterile PBS to remove any unbound growth factor. Beads were then re-suspended in sterile PBS and 10–15 beads bearing IGF1 or BSA were transferred onto each well of Ishikawa cells in 96-well plate as previously described (Kang et al., 2014). After 24–48 h of co-culture, cells containing beads were washed with PBS and fixed with 4% PFA. The number of attached beads was counted and analysed by normalizing to the total number of beads transferred.

### Western blotting
Following appropriate experimental treatment, cells were washed in PBS and scraped into RIPA buffer. 30 µg of protein was loaded into each well of 8% SDS-polyacrylamide gels and protein was separated electrophoretically by molecular mass. The separated proteins were then transferred onto PVDF membrane (Millipore) and blocked with 5% BSA in TBS with Tween-20 at room temperature for 1.5 h. Membranes were then probed with rabbit anti-phospho-IGF1R antibody (1:1000; Product number 3027; Cell Signaling Technologies), or a rabbit antibody against the β subunit of IGF1R (C20; 2 µg/ml; Santa Cruz Biotech) at 4℃ overnight, washed with TBS with Tween-20, and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit-IgG antibody at room temperature for 1 h. The protein was exposed to the enhanced

chemiluminescence system (Amersham) and then to Amersham or Kodak hyperfilm. Blots were stripped and reprobed with mouse-anti-β-actin (4.5 µg/ml; Sigma) or rabbit anti-GAPDH (1:1000; Cell Signaling Technologies) antibodies to control for protein loading. ImageJ (National Institute of Health, Bethesda, MD) was used to quantify bands comparing to loading control.

### Assessment of miR-145–IGF1R interaction

pmiR-GLO Dual-Luciferase miRNA Target Expression Vector (Promega) was used to demonstrate the interaction between miR-145 and IGF1R. The 3′ untranslated region (UTR) of IGF1R was cloned into the multiple cloning site (MCS) in the 3′UTR of the firefly luciferase gene. The principle of the vector is such that if endogenous miRs bind to the 3′UTR following introduction into cells, translation of firefly luciferase will be reduced.

### IGF1R 3′UTR luciferase reporter constructs

Two 42 base pair (bp) oligonucleotides and complementary antisense strands were designed using TargetScan (http://www.targetscan.org) to correspond to the highly conserved region of the IGF1R 3′UTR to which miR-145 is predicted to bind (3804–3810 bp; supplementary material Table S1). Pme1 and Xba1 overhangs were incorporated to ensure correct orientation of the oligonucleotide from 5′ to 3′. A Not1 restriction site was included, to act as an internal restriction site to confirm successful ligation. A scrambled version of the first predicted binding site was included as a control (supplementary material Table S1).

Prior to ligation, the pmiR-GLO Dual-Luciferase miRNA Target Expression vector was linearized with Pme1 (New England Bio Labs) and Xba1 (Roche) restriction enzymes to ensure complementarity with oligonucleotide overhangs. 1 µg of vector was incubated with 1 µl of each restriction enzyme and 2 µl of 10× CutSmart Buffer 4 (New England Bio Labs) overnight at 37℃, samples were then heated to 65℃ for 15 min to deactivate enzymes prior to electrophoresis. The linearized vector underwent gel electrophoresis on a 1% agarose gel and was then extracted using the Qiagen gel extraction and purification kit. Prior to ligation with the linearized vector, complementary oligonucleotides were annealed using Oligo Annealing Buffer (Promega) following the manufacturer's instructions. 4 ng of annealed oligonucleotides were incubated with 50 ng of linearized vector, 2 µl of T4 DNA ligase (New England BioLabs) and 1 µl of 10× T4 DNA ligase buffer (New England BioLabs) at 4℃ overnight. Following ligation the vector, which contains an ampicillin resistance gene, was transformed into XL-10 Gold Ultra competent cells (Aligent Technologies) using heat shock as per the manufacturer's instructions. Cells were then plated onto ampicillin-containing agar plates and incubated at 37℃ overnight. Colonies were selected and grown overnight in LB medium containing ampicillin at 37℃ and the vector was purified using the Qiagen mini prep kit (Thermoscientific). Not1 digestion was performed to confirm successful ligation of the IGF1R and scrambled 3′UTR binding sites.

### Luciferase reporter assay

Following successful ligation, vectors (50 ng) containing miR-145, or IGF1R 3′UTR sites and pSV-β-Galactosidase Control Vector (100 ng; Promega) were transfected into Ishikawa cells alone or in combination with miR-145 or non-targeting miR mimics (100 nM) using DharmaFECT2 as described above. Untreated and mock-transfected samples and the pmiR-GLO vector were included as controls. 24 h after transfection cells were washed twice in PBS and lysed with passive lysis buffer (Promega). Levels of Firefly luciferase were read using the Orion L Microplate Luminometer (Titertek) using the luciferase reporter assay (Promega) according to the manufacturer's instructions. Levels of β-galactosidase were measured using the mammalian β-galactosidase assay kit (Pierce, Thermoscientific). Firefly luciferase was normalized to β-galactosidase in each sample to control for transfection efficiency.

### miR-binding site target protector

Target protectors (purchased from Qiagen) were designed to correspond to the region of the IGF1R 3′UTR (3804–3810 bp) to which miR-145 is predicted to bind (5′-TACCTACCGGTTTCCAC<u>AACTGGA</u>TTTCT-ACAGATCATTC-3′, where the underlined region represents the seed sequence). BLAST analysis demonstrated that the target protector did not have any homology to any mammalian genes, other than IGF1R. The target protector is therefore specific and allows effective blocking of miR-145–IGF1R interaction without influencing binding of miR-145 to its other targets (Stanton and Giraldez, 2011). Effects of miR-145 altered in the presence of the target protector, are thus attributable to interaction between miR-145 and IGF1R. A scrambled target protector was designed as a control (sequence, 5′-TACCTACCGGTTTCCAC<u>AAAAGGA</u>T-TTCTACAGATCATTC-3′, Δ3804–3810 bp). Ishikawa cells were transfected with scrambled or IGF1R target protectors (100 nM) individually or in combination with miR-145 or non-targeting mimics (50 nM). 48 h later, IGF1 or BSA-coated beads were added to the cells and 24–48 h later the ability of miR-145 to influence IGF1R expression and bead attachment were assessed.

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